ENHANCING THE CATALYTIC POTENTIAL OF NITRILASE FOR STEREOSELECTIVE NITRILE HYDROLYSIS

A THESIS

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By

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CERTIFICATE

This is to certify that the work embodied in this thesis entitled **"Enhancing the catalytic potential of nitrilase for stereoselective nitrile hydrolysis"** is a piece of research work done by **Mr. Brajesh Barse** under my guidance and supervision for the degree of Doctor of Philosophy of Bundelkhand University, Jhansi, U.P., India.

To the best of my knowledge and belief the thesis:

1. Embodied the work of the candidate herself.
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S. Researcher has put in over 200 days of attendance in the laboratory to complete the work.

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DECLARATION

I hereby declare that the thesis entitled **"Enhancing the catalytic potential of nitrilase for stereoselective nitrile hydrolysis"** being submitted for the Degree of Doctor of Philosophy in Microbiology of Bundelkhand University, Jhansi U. P. is an original piece of work done by me and to the best of my knowledge and belief, it is not substantially the same one which had already been submitted for the degree of any other academic qualification at any other university or examining body in India or abroad.

Date: 29/5/09
Place: B.U. Jhansi

Brajesh Barse

To My Wife

For giving me inspiration

&

My Mentor

For his encouragement

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I think if any of us honestly reflects on who we are, how we got here, what we think, we might do well and so forth, we discover a debt to others that spans written history. The work of some unknown persons makes our lives easier everyday. I believe it's appropriate to acknowledge all of these unknown people, but it is also necessary to acknowledge those people we know have directly shaped our lives and our work.

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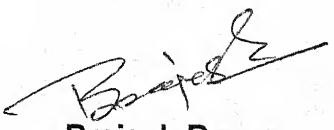


Brajesh Barse

CONTENTS

ABBREVIATIONS

µg	Microgram
µl	Microlitre
ACN	Acetonitrile
ATCC	American type culture collection
Conc.	Concentration
DKR	Dynamic kinetic resolution
DO	Dissolved oxygen
EA	Enzyme activity
Fig.	Figure
g	Gram
g/l	Gram per litre
h	Hour
kDa	Kilodalton
K_La	Volumetric oxygen transfer coefficient
M	Molarity
mg	Milligram
min	Minute
ml	Millilitre
ml/min	Millilitre per minute
mM	Millimolar
MTCC	Microbial Type Culture Collection
N	Normality
^oC	Degree Celsius
OD	Optical density
rpm	Revolutions per minute
sec	Seconds
Sp. Act.	Specific activity
U	Enzyme Units
v/v	Volume by volume

vvm	Volume of air per volume of media per minute
w/v	Weight by volume

LIST OF FIGURES

LIST OF TABLE

ABSTRACT

Nitrilases constitute an important class of hydrolases, however, cheap and ready availability of enzyme sources and stability of the existing enzymes limit their practical applications. Nitrilase is a key enzyme in the production of mandelic acid an important pharmaceutical intermediate. Mandelic acid used as a chiral resolving agent and used for the synthesis of anti-tumor and anti-obesity agents, it also possesses antifungal activity, broad spectrum β-lactamase inhibitor and anti-oxidative properties. Production of mandelic acid through biocatalysis gives inherent advantages of this process over expensive chemosynthesis. The present work has concerted to address the production and utilization of a highly enantioselective recombinant nitrilase for the hydrolysis of racemic mandelonitrile to (*R*)-mandelic acid. Inducer replacement studies led to the identification of lactose as a suitable and cheap alternative to the costly IPTG. Effects of medium components, various physico-chemical, and process parameters (pH, temperature, aeration, and agitation) for the production of nitrilase by engineered *E. coli* were optimized and scaled up to a laboratory scale bioreactor (6.6 l) the production of mandelic acid with use of nitrilase enzyme by developing immobilized, stable and reusable biocatalyst. Immobilized cells of recombinant *Escherichia coli* was employed for hydrolysis of mandelonitrile to *R*-(-)-mandelic acid. The immobilization of recombinant *Escherichia coli* was done using matrix entrapment method using sodium alginate as matrix. Immobilization parameters and the reaction conditions for the mandelic acid production have been optimized in shake flask. The optimal values for immobilization were matrix concentration 2% (w/v), bead diameter 4.30 mm, reaction pH 8, reaction temperature 40°C, cell concentration 20 mg/ml, reaction time 60 min and substrate concentration 50 mM. This optimization study approach led to increase the conversion of mandelic acid to 95%.

Introduction

&

Review of Literature

1. Introduction

Biocatalysis involves the utilization of enzymes and/or whole cells to bring about various chemical reactions. The core technology concerns harnessing catalytic power of enzymes for synthesizing commercially important and useful products. The use of enzymes in the food industry is historically known, but their utilization by the pharmaceutical and fine chemical industry is rapidly expanding only in recent times [Huisman, G. W., and D. Gray. 2002]. This has been attributed to recent notable advances in molecular biology, high throughput screening, instrumentation and engineering. These advances have improved the access to biocatalysts, increased their stability and broadened their specificity [Zaks, A. 2001]. Although there are still some myths about biocatalysts, which restrict their use in the industry [Faber, K. 2000, Rozzell, J. D. 1999], these are being rapidly demystified and biocatalysts are increasingly seen as being highly promising for sustainable and selective production of complex chemical entities, active pharmaceutical ingredients and their intermediates [Schoemaker, H. E., D. Mink, and M. G. Wubbolts. 2003]. With the potential of biocatalysts being rapidly grasped by the world, the field is growing at a brisk rate utilising the advances in technology that are being tailored to meet the specialized applications that call for its use.

This is especially so for the synthesis of chiral drugs. Homochiral and single enantiomer drugs are gaining importance in recent years due to the increased pressure on the manufacturers by the regulatory authorities and the market conditions [Borman, S. 1992]. This is due to the fact that only a single enantiomer may have the desired chemical or pharmacological property. When the racemate is marketed then the unwanted enantiomer acts as 'ballast', which only increases the amount of the product without actually being of any use. In certain cases the unwanted isomer may also have deleterious effects. The major advantages of using enantiomerically pure drugs include reduction of the total administered dose, enhanced therapeutic window, reduction of variability and a more precise estimation of dose response relationship [Caldwell, J. 1999].

Biocatalytic processes differ from conventional chemical processes mainly in terms of enzyme kinetics, protein stability under technical conditions and catalyst features. The advantages of biocatalysts include their *chemo-*, *regio-*, and *stereo-* selectivity as well as substrate and functional group specificity. In addition, they also reduce the complications of isomerization, racemization, epimerization and rearrangements that are common with chemical reactions [Ward, O. P., and A. Singh. 200]. Also biocatalysts function at mild reaction conditions like ambient temperature, atmospheric pressure and have a low side product generation. These make the processes environment friendly thus rendering them the ideal agents for 'green chemistry'. Along with the environmental concerns being taken care of, it also makes commercial sense as there is a reduction in the steps needed for the synthesis of the pure compound as well in effluent generation and treatment. Enzymes nowadays have been found to be equally efficient in both aqueous and organic media, thus increasing the scope of their utility [Rozzell, J. D. 1999, Schmid, A. et al 2001.]. The field is also rapidly expanding due to the better exploitation of available natural diversity, use of high throughput screening coupled with genetic manipulation, cloning techniques [Goddard, J.-P., and J.-L. Reymond. 2004], protein engineering and other directed evolution methods [Jaeger, K. E., and M. T. Reetz. 2000] which has made it possible to design an enzyme for a commercial process [Robertson, D. E., and B. A. Steer. 2004, Turner, N. J. 2003]. As a result biocatalyst based strategies are considered to be ecologically and economically viable alternatives for the synthesis of fine chemicals, pharmaceuticals, agrochemicals and even bulk chemicals, in preference to traditional organic synthesis.

Mandelic acid and its uses

Mandelic acid is α-hydroxy acid obtained naturally from several *Prunus* species in their glycosidic form, especially from bitter almonds. It is an important pharmaceutical intermediate as well as a specialty chemical. Mandelic acid has use in the commercial synthesis of semi-synthetic cephalosporins and penicillins [Terreni, M. et al, 2001]. It is also used as a chiral resolving agent [Kinbara, K., K. Sakai, Y. Hashimoto, H. Nohira, and K. Saigo. 1996] and for the synthesis of

anti-tumor [Surivet, J.-P., and J.-M. Vatele. 199] and anti-obesity agents [Mills, J., K. K. Schmiegel, and W. N. Shaw. 198]). It has also been shown to possess antifungal [Kope, H et al, 1991], broad spectrum β-lactamase inhibitor [Mollard, C et al, 2001] and anti-oxidative properties [Ley, J. P., and H. J. Bertram. 2001]. A mandelic acid condensation polymer is also being investigated for prevention of entry of human immunodeficiency virus (HIV) and herpes simplex virus through sexual transmission [Zaneveld, L et al, 2002]. Mandelic acid used alone or in combination with vitamins, are marketed as beneficial cosmeceutical agents for their antibacterial effects and improvement in photo-aged skin, acne, abnormal pigmentation, and skin texture.

Different routes to optically pure mandelic acid

(*R*)-(-)-mandelic acid is presently produced by the resolution of racemic mixture by chiral amines [Gatterman, L., and H. Wieland. 1927]. It is also enzymatically produced via lipases [Kimura, M., A. Kuboki, and T. Sugai. 2002], esterases [Mori, K., and H. Akao. 1980], glyoxylases [Patterson, M., R. Szajewski, and G. Whitesides. 1981], mandelate dehydrogenase [Yamazaki, Y. et al, 1986] and mandelate racemase [Yamamoto, K. et al, 1991, Strauss, U et al, 1999]. The drawback of these enzymatic methods includes the use of expensive co-factors, operational stability and production economics [Yamamoto, K. et al, 1991, Kaul, P. et al, 2004]. These drawbacks are the prime reasons, which prevent the setting up of a commercially feasible industrial scale synthetic process. Nitrilase mediated hydrolysis on the other hand can be commercialized on a large scale, as it does not involve costly cofactors. This process prevents the additional step of separation of the non-reactive isomer, which would add to the costs. Hence the process is comparatively more economical.

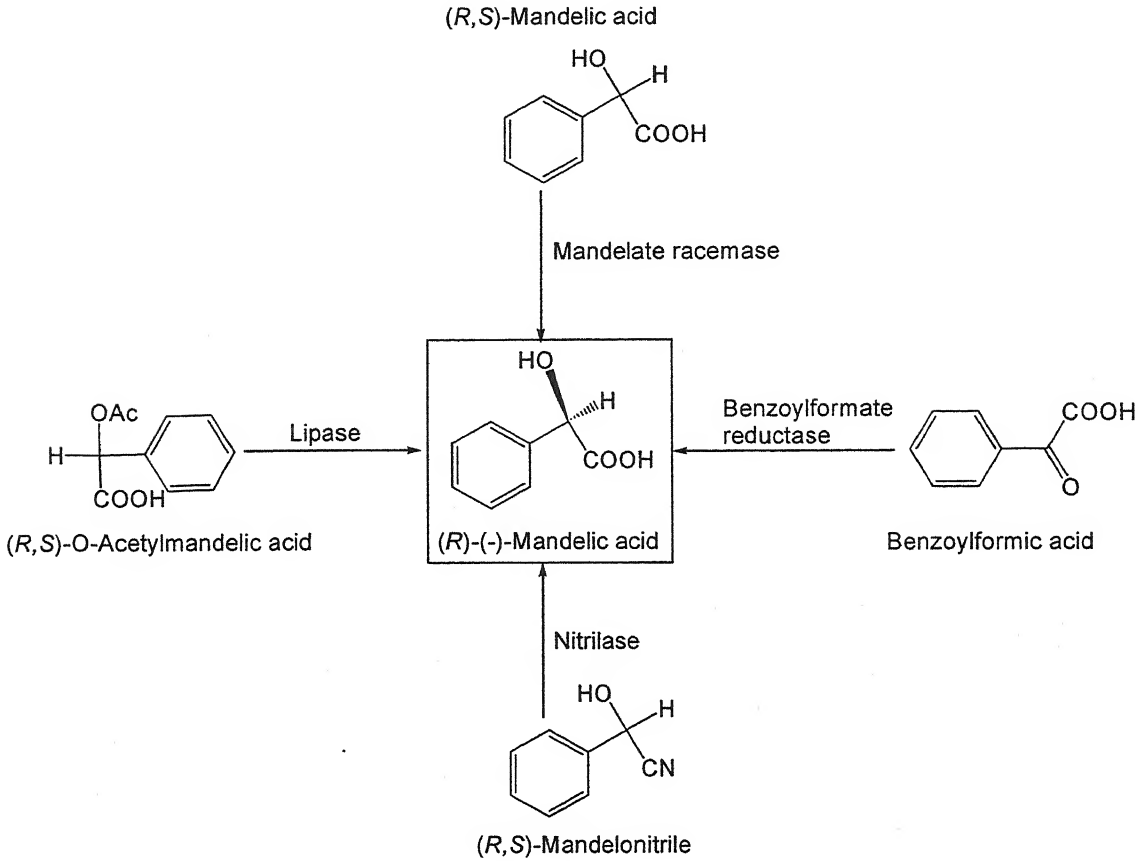


Figure 1. Different routes of enzymatic synthesis of (*R*)-(-)-mandelic acid

Kinetic and dynamic resolution of mandelonitrile

One of the important applications of nitrilases involves the enantioselective hydrolysis of racemic mandelonitrile to (*R*)-(-)-mandelic acid. This is a kinetic resolution process where the enzyme selectively hydrolyses one enantiomer of the racemate while the other remains untouched [Turner, N. J. 2003, Sheldon, R. 1993] the disadvantage of this process is that a maximum of 50% theoretical yield can be obtained as only one enantiomer is transformed. Also an additional step is required for the separation of the unreacted isomer. An offshoot of this process is that of dynamic kinetic resolution (DKR) where the unreacted substrate enantiomer is converted to the racemate form and then the reaction proceeds giving a theoretical yield of 100%. Thus there is a generation of high

yield and high enantiomeric excess (ee). In general DKR involves two reactive systems, i.e. a resolution system and a racemization system. At slightly alkaline pH, mandelonitrile undergoes spontaneous degradation to benzaldehyde and hydrocyanic acid. This can constitute an efficient DKR, where the unreacted (*S*)-mandelonitrile undergoes simultaneous degradation and racemization to give theoretical 100% conversion to (*R*)-(-)-mandelic acid by nitrile hydrolysis. The arylacetonitrilases of several organisms including *Alcaligenes faecalis* ATCC 8750 [Yamamoto, K. et al 1991] and *Pseudomonas putida* MTCC 5110 [Kaul, P. et al, 2004] have been reported to bring about the enantioselective hydrolysis of mandelonitrile to (*R*)-(-)-mandelic acid by this dynamic process. But the main drawbacks with these organisms are their slow growth rate, and very less information is available regarding their cultivation at large scales. On the other hand a recombinant *Escherichia coli* BL21 (DE3) system is supposed to be a good candidate for the biotransformation, taking into account its higher growth rate, faster reaction rate and higher stability.

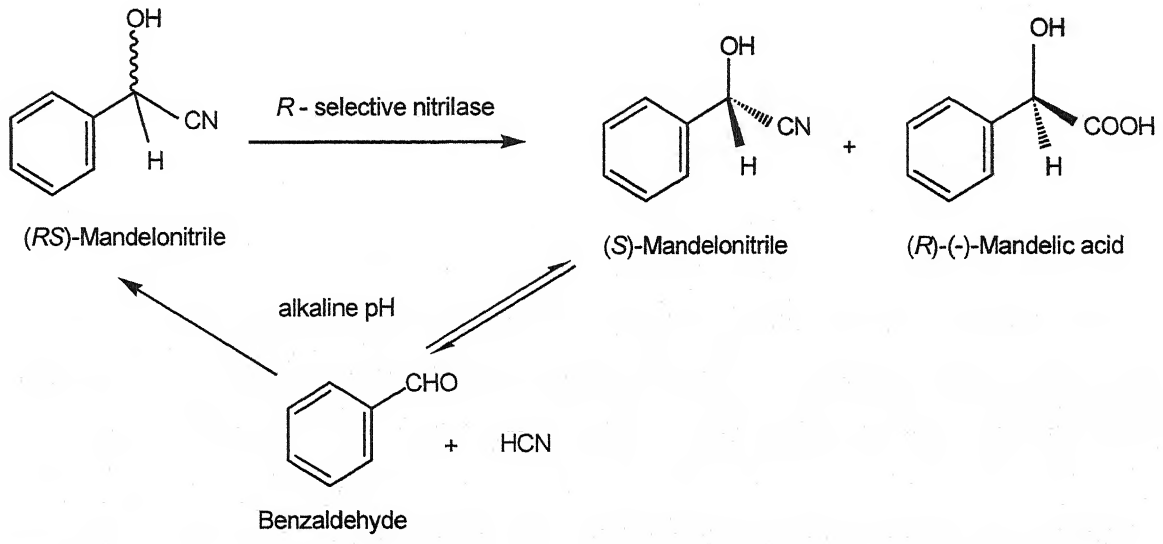


Figure 2. Mechanism of dynamic kinetic resolution of mandelonitrile hydrolysis.

Scale up

Scale up involves the transfer of a process from smaller scale to higher scale. A successful scale up is a process which been designed and built to give a predictable increase in the production capacity. The fundamental idea is that the optimal physiological conditions obtained at small scale studies should be maintained at larger scale by controlling the different environmental parameters. However there is often a decrease in productivity as compared to shake flask. This is because in spite of the best efforts, due to the higher volumes in large scale, the gradient in terms of nutrient and substrate concentration, dissolved oxygen (DO) concentration, pressure, temperature, etc. are generated and the same physiology of microbe is difficult to obtain. As a result of this, there is a vast difference in the environment in a shake flask and the larger vessel. This change in the environment prompts the organism to respond in manner different from what it had been exhibiting. These responses are often unknown and unpredictable. Hence it can be said that the every process is unique in its own way [Reisman, H. B. et al 1993]. As a consequence, the process has to be developed in a manner such that the process parameters are maintained to give maximum productivity at that particular scale. These parameters may be physico-chemical or biological in nature. Valid relationships which remain true irrespective of the scale can however be developed only for the former. As even subtle changes in these parameters can affect the productivity enormously hence due care should be taken while fixing and optimizing these parameters [Hoshobuchi, M. et al, 2003, Thiry, M et al 2002]. Optimization is carried out by changing one parameter at a time and looking for the effect generated by this. Also statistical methods can be used wherein different parameters are changed simultaneously and the effect is visualised by means of statistical analysis. However, before doing this, it is essential to identify the variables or the parameters that could affect the conditions.

Immobilization

The use of a relatively expensive catalyst as an enzyme requires, in many instances, its recovery and reuse to make an economically feasible process [Chibata, I., Tosa,T., and T Sato. 1986]. The use of enzymes in industrial applications has been increasing day by day. Despite of number of advantages of enzymes over chemical catalysts their applications has been limited, mainly because of high cost of enzyme production and purification, availability in small amounts and their instability in normal conditions. Nowadays there are many approaches with which the use of enzymes can be increased and one of the best method is it immobilize the enzymes or whole cells [Ishige, T., K. Honda, and S. Shimizu. 2005].

Moreover, the use of an immobilized enzyme permits to greatly simplify the design of the reactor and the control of the reaction the simple filtering of the enzyme stops the reaction; it is possible to use any kind of reactor, etc. Thus, immobilization is usually a requirement to the use of an enzyme as an industrial biocatalyst, and is the simplest solution to the solubility problem of these interesting biocatalysts. However, the idea of enzyme reuse implicitly means that the stability of the final enzyme preparation should be high enough to permit this reuse. Therefore, the enzyme needs to be very stable or to become highly stabilized during the immobilization process to be a suitable process[Chibata, I., Tosa,T., and T Sato. 1986].

Generally immobilization means to render the movement whether it is an enzyme or whole cell. In scientific language, it is an association of the biocatalyst or whole cell with an insoluble matrix, so that it can be retained in a proper geometry or shape for its regular use. It can also be defined as the physical confinement or localization of enzymes with the retention of their catalytic activity and can also be used repeatedly and continuously [Hartmeier, W.1985]. These localized enzymes can withstand harsh environmental conditions of pH, temperature and organic solvents etc. Immobilization renders the enzyme loss with the flow of the liquid carrying reagents and products. Therefore, the use of

the localized enzymes helps in the development of continuous processes [Katchalski-Katzir, et al. 1993].

The important aspect in immobilization is the selection of the support used for immobilization. The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization. The choice of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its ultimate application. Prior to immobilization the supports are activated for the attachment of the enzyme. There are various types of activation procedures like in case of polysaccharides; activation is most commonly via derivatization of available hydroxy functions using reagents such as cyanogen bromide, cyanuric chloride, sodium periodate or benzoquinone. The activated support is then covalently linked to the enzyme, most commonly via direct reaction with available amino functions, e.g. lysine residues[Tischer, W., and V. Kasche. 1999].

Techniques for immobilization

Covalent bonding

This is an extensively used technique for the immobilization of enzymes. The enzymes are covalently linked to the support through functional groups such as amino, carbonyl and phenolic groups of tyrosin, thiol, indole, sulfhydryl, hyroxy, imidizole, threonine etc which are not essential for the enzyme activity.

Enzymes like glucose oxidase, peroxidase etc has been immobilized using this technique. Generally the lysine residue has been found for covalent bonding since they are found to be rarely involved in the active sites of enzymes. The binding force involved in this method is so strong that no leakage of enzyme occurs with the changing conditions but this may alter the conformational structure and the active site of the enzyme, resulting in the loss of the activity. Large scale process using such approach has been used for the preparation of sugar [Chibata, I., Tosa,T., and T Sato. 1986, Hartmeier, W.1985, Katchalski-Katzir,E. 1993, Tischer, W., and V. Kasche. 1999].

Cross-linking

Enzymes or whole cells can also be immobilized through chemical cross linking using homo as well as hetero bifunctional cross linking agents. Immobilization is achieved by intermolecular cross liking of the protein, either to the other protein molecule or to the functional group on an insoluble support matrix, but the latter one is preferred because former is expensive. The most commonly used agent for cross linking is glutaraldehyde because it contains large concentration of lysozyme which gets co-immobilized thus imparting bacteriolytic property to support. This method is best used in conjugation with other methods like adsorption, entrapment etc. Since it involves the formation of covalent bonds mainly with the support so there are very less chances of desorption but this method is expensive and leads to significant loss of the active site of the enzyme [Chibata, I., Tosa,T., and T Sato. 1986, Hartmeier, W.1985, Katchalski-Katzir,E. 1993, Tischer, W., and V. Kasche. 1999].

Adsorption

Adsorption technique is the simplest way of preparing immobilized enzymes. The method is based on the non-specific physical interaction between the enzyme protein and the surface of the matrix. Under the appropriate conditions of pH and ionic strength, the enzyme is mixed with suitable adsorbent which is followed by washing with the loosely bound enzyme after suitable incubation period. This will produce directly usable immobilized enzymes. The interactions involving the method are hydrophobic forces and several salt links per enzyme molecules. The adsorbents used are ion-exchange matrices, porous carbon, clays, glasses and polymeric aromatic resins etc. this method has been successfully adapted in industry for the resolution of racemic mixture of amino acids using amino acid acylate[Chibata, I., Tosa,T., and T Sato. 1986, Hartmeier, W.1985, Katchalski-Katzir,E. 1993, Tischer, W., and V. Kasche. 1999].

Entrapment

This method id based on the localization of an enzyme within the lattice of the polymer matrices or membrane. It allows the retention of the protein within the lattice while allowing the penetration of the substrate. It differs from other method by the non-binding of the enzyme to the gel matrix or membrane. Entrapment is mostly used for the immobilization of cells but not for enzymes. Natural polymers like agrose, agar, gelatin etc and synthetic polymers like polyacrylamide, polyurethane, prepolymers can be used for entrapment. Since there is no bond formation between proteins and the matrix or support, there are no chances of enzyme disruption or change in conformation [Chibata, I., Tosa,T., and T Sato. 1986, Hartmeier, W.1985, Katchalski-Katzir,E. 1993, Tischer, W., and V. Kasche. 1999].

Effect of immobilization on microbial cells

Alterations in cell growth, physiology and metabolic activity may be induced by cell immobilization, of both yeast and bacteria species. It has been generally observed that it is difficult to predict the type and magnitude of metabolic changes possible through immobilization. A number of parameters have been considered responsible for these alterations, such as mass transfer limitations by diffusion, disturbances in the growth pattern, surface tension and osmotic pressure effects, reduced water activity, cell-to cell communication, and changes in the cell morphology, altered membrane permeability and media components availability. Comparative studies on immobilized and free cells reported effects on activation of yeast energetic metabolism, increase in storage polysaccharides, altered growth rates, increased substrate uptake and product yield, lower yield of fermentation by-products, higher intracellular pH values and increased tolerance against toxic compounds [Ratledge, C., and B. Kristianzen. 2001, Fogler, H. S. 1999]

Advantages of whole cell immobilization

1. Prolonged activity and stability of the biocatalyst.
2. Higher cell densities per unit bioreactor volume
3. Increased substrate uptake and yield improvement.
4. Increased tolerance to high substrate concentration and reduced end product inhibition.
5. Easier product recovery through reduction of separation and filtration requirements, thus reducing cost for equipment and energy demands.
6. Regeneration and reuse of the biocatalyst for extended periods in batch operations, without removing it from the bioreactor.
7. Reduction of risk of microbial contamination due to high cell densities and fermentation activity.
8. Ability to use smaller bioreactors with simplified process designs and therefore lower capital costs.
9. Reduction of maturation times for some products.

Disadvantages of whole cell immobilization

1. Low volumetric productivity
2. Unwanted reactions with impure preparations
3. Poor operational stability and high cost

Review of Literature

The research in this thesis lies within the scope of Biocatalysis: which deals with synthetic methods that exploit nature's catalysts, for the preparation of valuable products.

Modern biotechnology uses, amongst others, enhanced micro-organisms like yeast, moulds, and bacteria as 'cell factories,' along with the enzymes derived from them, to produce a variety of goods. Biotechnology has found its entry into medicine (red) and agriculture (green), and now a new wave of modern biotechnology is gaining momentum – 'white biotechnology' is the application of nature's toolset to industrial production [Lorenz et al, 2005] [Young et al, 2003]. White biotechnology has become much more broadly applicable due the development of genetic manipulation techniques. Multiple enzyme variants, for example, can now be created at high speed, which are then screened for fit with the desired application [Kuchner et al, 1997]. Although enzymes have been used on an industrial scale since the 1950s, full acceptance of their role has been realized only recently, with the lead coming from the fine chemicals industry. Many of the drawbacks perceived by the process engineers, such as low yield and throughput, high dilutions, limited enzyme availability and low stability, have largely disappeared. It is now accepted that water may be a suitable medium for industrial processes while at the same time enzymes are being modified in such a way that they can be used in the organic media with which chemists are more familiar [Klibanov et al, 2001].

1.1. Towards Biobased Values

By taking the advantage of technological breakthroughs in biotechnology, governments, industries and nongovernmental organizations (NGOs) are developing strategies and action plans to avert looming social and economic crisis [Carole et al, 2004]: This is the case in UK, USA, Japan and many other industrialized nations. Dwindling energy resources, political instability in oil and

gas exporting countries and increasing competition for resources jeopardize security and prosperity specially in booming economies like India. It is felt that biotechnology applied to at an industrial scale could be a smart and feasible way of alleviating some of these forecasted effects and might also help to maneuver economies and societies into a sustainable, safer and greener future [Herrera et al, 2004] [Dale et al, 2003]. An estimate by McKinsey & Company shows that biotechnology could be applied in the production of 10 to 20 % of all chemicals sold by the year 2010. Even for the traditional mainstay of the chemical industries, the polymer market (typical bulk-volume products) McKinsey predicts that biotechnology might account for upto 10 % of the output [Ariens EJ 2003]. This estimate is based on the analysis of technology and market trends as well as on an inventory of current R&D activities. The market share was calculated bottom-up by estimating the potential for white biotechnology applications amongst key chemical products and product groups. Starting with the chemical industry, white biotechnology will make inroads into a number of other industries. For example, enzymes will transform production processes in the pulp and paper industry, and new polymers will find multiple applications in the automotive and consumer industries. In response to this assessment, the initiatives of leading industrialized nations have instigated industrial biotechnology as one of the key drivers of growth. The European Chemical Industry Council and the European Association for Bioindustries (EuropaBio) (www.europabio.org) have jointly launched the *European Technology Platform for Sustainable Chemistry* [Davies N et al, 2004] in 2004. SusChem is intended as a tool to strengthen and boost the competitiveness of the European Chemical Industry by integrating biotechnology into industrial research. Clearly, this joint European initiative does not come without 'stimulating' precedent from the forward-looking peers in US or Japan, and leading European stakeholders (among them DSM, Degussa, BASF, Henkel, Novozymes and Genencor) have urged policy makers to act to ensure a favorable competitive position.

1.2. Chirality and Biological Activity

Chirality has now become a major theme in the design, discovery, development, launching and marketing of new drugs [Eichelbaum et al, 1996]. Until the recent past, pharmacopoeia was dominated by racemates, but since the emergence of new technologies in the 1980s that allowed the preparation of pure enantiomers in significant quantities, the awareness and interest in the stereochemistry of drug action has increased. Considering the molecular components of living organisms are largely chiral, it is not at all surprising that chirality plays a dominant role in their interaction with bioactive substances. The advances in stereoselective bioanalysis led to a new awareness of the importance of stereoselective pharmacodynamics and pharmacokinetics, enabling the differentiation of the relative contributions of enantiomers to overall drug action. When one enantiomer is responsible for the activity of interest, its paired enantiomer could be inactive, possess some activity of interest, be an antagonist of the active enantiomer or have a separate activity that could be desirable or undesirable [Shah et al, 2003] [Caldwell et al, 1999] [Caldwell et al, 2001]. In case of single enantiomeric drugs when one enantiomer (eutomer) is responsible for the activity of interest, its paired enantiomer (distomer) could be inactive, an antagonist of the eutomer, or may have a separate activity that could be undesirable and sometime hazardous [Ariens et al, 1984]. Considering these possibilities, there appears to be major advantages in using stereochemically pure drugs, such as a reduction of the total administered dose, enhanced therapeutic window, reduction of intersubject variability and a more precise estimation of dose–response relationships [Davies et al, 2003]. These factors have led to an increasing preference for single enantiomers in both industry and regulatory authorities. Regulatory control of chiral drugs began in the US with the publication of formal guidelines on the development of chiral drugs in a document entitled Policy Statement for the Development of New Stereoisomeric Drugs in 1992 [Administration et al, 1992; Strong et al, 1999; Witte et al, 2005] and was followed in the European Union (EU) in 1994 by Investigation of Chiral Active Substances [Products et al, 1993]. Applicants must recognize the occurrence of

chirality in new drugs, attempt to separate the stereoisomers, assess the contribution of the various stereoisomers to the activity of interest and make a rational selection of the stereoisomeric form that is proposed for marketing [Agranat et al, 2002]. In addition to these, chiral effects on therapeutics are exploited in two different ways: i) by developing better single enantiomeric version of already approved racemic drugs (racemic switching) [Agranat et al, 2002] and ii) enantiomeric drug discovery. Considering all these facts, there appears to be major advantages in using enantiomerically pure drugs, such as reduction of the total administered dose, enhanced therapeutic window, reduction of variability and a more precise estimation of dose response relationship [Caldwell et al, 1999]. Racemic switching also helps in extending the market and patent protection of these improved chemical entities (ICEs). Worldwide sales of chiral drugs in the single enantiomeric form are continuously growing at a brisk rate and expected to reach $ 160 billion by 2005 [Caner et al, 2004; Rouhi et al, 2003]. As more and more technologies become available for production of enantiopure chiral compounds, the demand for such compounds continues to rise, primarily for use as pharmaceuticals, but also as flavor and aroma chemicals, agrochemicals and speciality fine chemicals.

1.2.1. Accessing the Chirality Pool

A variety of technologies are now available for the efficient synthesis of a vast array of optically active compounds in enantiomerically pure form, which constitutes the chiral pool [Sheldon et al, 1993]. The rich diversity of chiral molecules that occur naturally as pure enantiomers and *de novo* fermentation of an inexpensive, abundantly available carbohydrate feedstock are the two major sources of chiral molecules in nature [Roper et al, 1991]. Enantiomerically pure compounds that are not readily available, can also be synthesized and thus form a part of the chiral pool. Synthetic routes to optically pure compounds include chemical manipulation of the already existing chiral molecules in nature, asymmetric synthesis of the chiral molecule from a prochiral substrate [Noyori et al, 1994] and resolution of racemates [Jacques et al, 1981]. Although revolutionary advances are being made in catalytic asymmetric synthesis,

resolution of racemates still remains the main method for industrial synthesis of pure enantiomers. Enantiomers can be separated from racemic mixtures either by preferential crystallization or diastereomeric crystallization or by kinetic resolution [Jacques et al, 1981]. Preferential crystallization can only be applied to conglomerates, and diastereomeric crystallization involves further derivatization of the diastereomeric salt that is being formed by the reaction with an optically active acid or base [Tosaka et al, 1986], leaving kinetic resolution as the most preferred way for the resolution of racemates. Alternatively, simulated moving-bed chromatography (SMB) can be used for the separation of the two enantiomers of a chiral molecule, which is feasible at all production scales. The use of non-enantioselective synthesis of racemic mixtures and simulated moving-bed enantiomer separation might make the development process of a new chiral drug substantially shorter and cheaper [Juza et al, 2000].

1.3. Present Status and Potential of Biocatalysis

Organic chemistry looks back on a continuous and fruitful development in the late 18^{th} century and which continues today. The uninterrupted development provided the methods for almost any synthesis, albeit ecologically and economically often still unfavourable. In contrast, the enzymatic toolbox for organic synthesis is much younger and not yet routinely used by synthetic organic chemists [Buckland et al, 2000]. The ideal situation for biotransformation would be that thousands of different stable enzymes preparations are commercially available. Most of the customer manufacturing organizations (CMOs) deal with pharmaceutical companies and are challenged with a broad spectrum of organic molecules. The main challenge faced by a CMO is to deliver the necessary quantity and quality of a key intermediate or active pharmaceutical ingredient (API) on time and with a cost-competitive process throughout the life cycle of the product. Therefore the activities of a process development chemist should aim towards the development of a commercial process where manufacturability, robustness and best fit to the plant will be established (Figure 3).

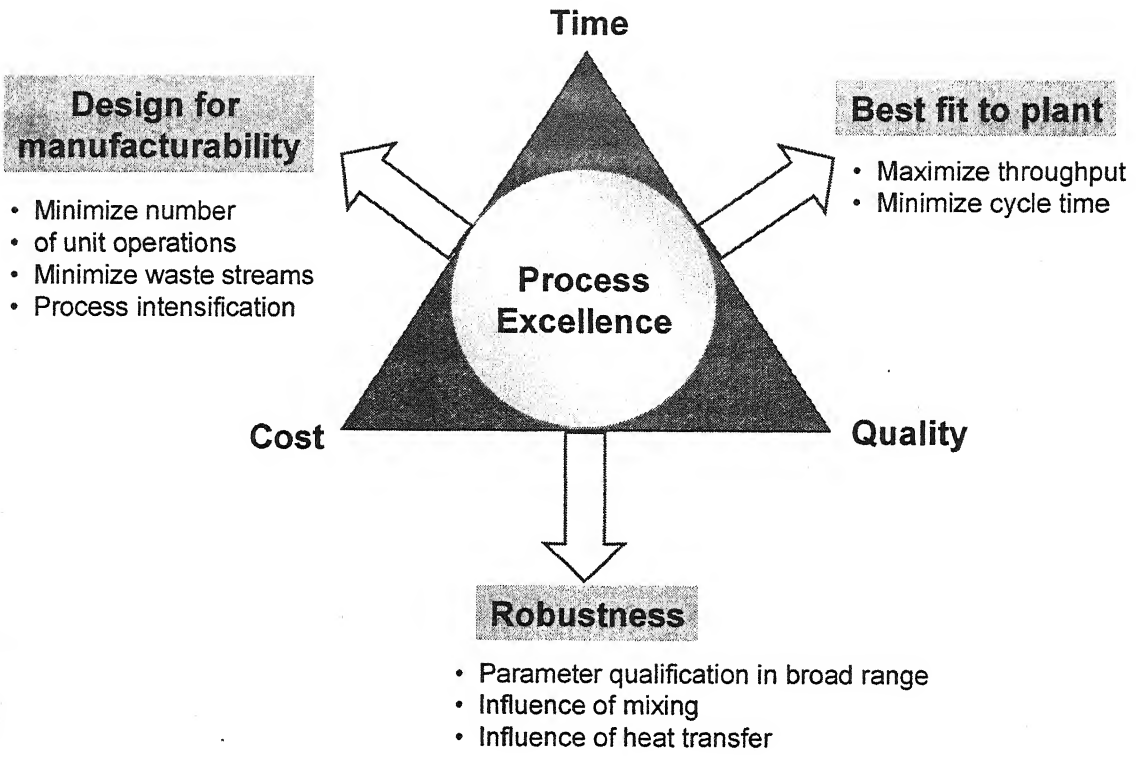


Figure 3. Major research and development challenges to build a profitable process

Often a bio option comes in question if the chemical arsenal cannot achieve the synthesis of the target molecules. This is so, because the appropriate strain and enzymes are often missing [Wells et al, 2006]. Creating a biocatalyst toolbox *de novo* or expanding it significantly does not come without a price. As funds are usually a limiting factor, the question arises of who would finance such a toolbox. It is not unusual for the biocatalysis division within a CMO to be viewed as an internal service provider. If this department wants to invest in such a library, it will not usually be willing or be able to spend substantial amounts of its budget betting on the odds of finding the perfect enzyme-substrate match. To overcome the strain shortage, creative technical as well as unusual nontechnical solutions are needed. The Swiss Industrial Biocatalysis Consortium is presently trying to establish an unconventional solution. Companies engaged in biotransformation have countless strains and enzymes that they will never use for a commercial process. As everybody in the industry has the same problem, the consortium

plans to share information on their library. In this way redundant strains might become valuable for both the strain receiver and the strain donor.

Efforts during the past decade have shown that there are very few barriers to the use of enzymes and whole cells as biocatalyst in organic synthesis [Faber et al, 1997]. Enzymes are remarkable catalyst, capable of accepting a wide array of complex molecules as substrates, and exquisitely selective, catalyzing reactions with unparallel *enantio*- and *regio*-selectivities [Schmid et al, 2001]. Biocatalysts require mild conditions (physiological pH and ambient temperature) and an environmentally attractive solvent (water) for their high activity. As a result, they can be used both in the simple and complex transformations without the need for tedious blocking and de-blocking steps which are common in *enantio*- and *regio*-selective organic synthesis. This affords in more efficient synthetic routes which are shorter, generate fewer by-products and hence, both economically and environmentally more attractive than conventional chemical catalysts [Alcalde et al, 2006; Tan et al, 2006]. All these attributes resulted in a myriad of applications, especially in the pharmaceutical and fine chemical sectors where high selectivity on complex substrate is critical [Pollard et al, 2007]. Biocatalytic processes differ from conventional chemical processes, owing mainly to enzyme kinetics, protein stability under technical conditions and catalyst features. The economic feasibility of a biocatalytic process depends on several factors including the type of the biocatalyst to be used around the specific reactor and hardware configurations. In analogy to chemical processes, most biocatalysts are used in immobilized form as heterogeneous catalysts that can be recovered and reused. There are also processes, however, based on homogeneously suspended cells or enzymes, which are sufficiently inexpensive to permit single use, without recovery or reuse. Currently much attention is being focused on the application of different catalytic methods – homogeneous, heterogeneous and enzymatic, as cleaner alternatives to the traditional organic synthesis [Shoemaker et al, 2003; Sheldon et al, 2004]. The challenge is to develop green, sustainable synthetic methodologies for the manufacture of fine chemicals and chiral building blocks for enantiopure pharmaceuticals. All these happen in an era when biocatalysis

takes a huge leap forward, especially due to the advancement in the associated fields like high throughput screening [Wahle et al r, 2001; Goddard et al, 2004], directed evolution [Arnold et al, 2001, Reetz et al, 2001], catalysis in organic solvents [Klibanov et al, 2001, Lee et al, 2002] etc. Advances in the recombinant DNA technology coupled with the progress in the field of protein engineering, has made it routine to manipulate enzymes such that they exhibit desired properties like substrate specificity, activity, selectivity, stability and so forth [Arnold et al, 2001, Bornscheuer et al, 2001]. Furthermore the development of an ever-increasing arsenal of immobilization techniques has provided effective methods for optimizing the operational performance, recovery and reuse of enzymes [Tischer et al, 1999; Cao et al, 2003]. Because of the remarkable achievements in these associated fields it is now possible to visualize, if not design, catalytic systems that approach the functional "ideal" [Cheetham et al, 1998; Burton et al, 2002]. A wealth of information has been accumulated on the practical issues for various enzymes, including the mechanisms by which they operate, their physico-chemical properties, the breadth of their substrate tolerance, the methods of their large-scale production and the reaction technology for their synthetic applications. Because of firm acceptance by the synthetic community for practical applications, enzyme based methodologies are now considered as an economically and ecologically viable alternative for the production of fine chemicals, pharmaceuticals, agrochemicals and even bulk chemicals, instead of traditional organic synthesis [Bull et al, 1999].

1.3.1. Nitrile hydrolyzing enzymes

Nitrile compounds are widespread in the environment because of their extensive use as feedstock, solvents, extractants, pharmaceuticals, drug intermediates (chiral synthons), pesticides (dichlobenil, bromoxynil, ioxynil, buctril) etc. In nature they are mainly present as cyanoglycosides, which are produced by plants and animals [Vetter et al, 2000, Wittstock et al, 2002]. Plants also produce other nitrile compounds such as cyanolipids, ricinine, phenylacetonitrile etc. Most

of the nitriles are highly toxic, mutagenic and carcinogenic in nature. The general toxicities of nitriles in humans are expressed as gastric diseases and vomiting (nausea), bronchial irritation, respiratory distress, convulsions, coma and osteolathrysm, which leads to lameness and skeletal deformities. Nitriles inactivate the respiration system by tightly binding to cytochrome-c-oxidase

[Solomonson et al, 1981]. Microbial degradation has been considered as an efficient way of removing highly toxic nitriles from the environment [Knowles et al, 1976]. Hydrolysis is the most common pathway for the microbial metabolism of nitriles. ``

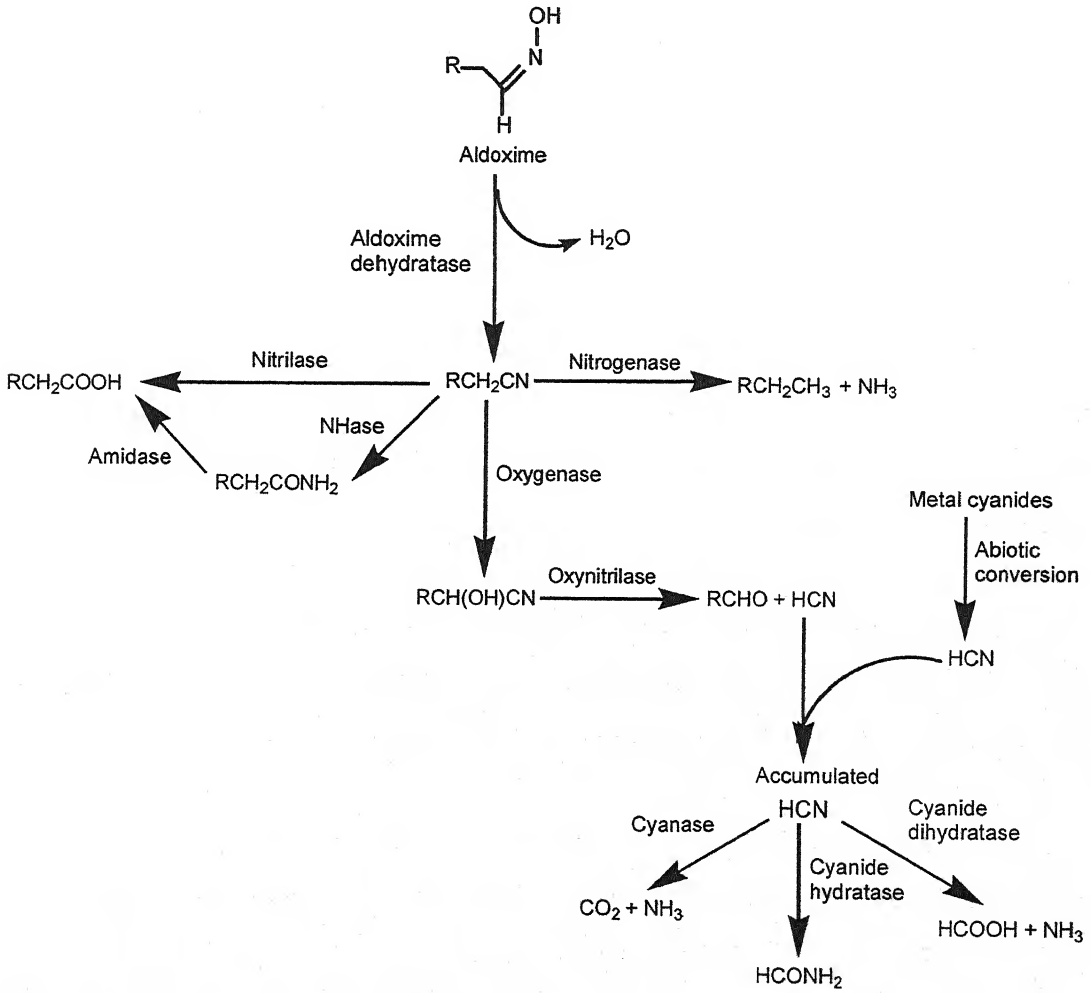


Figure 4. Enzymology of nitrile metabolism

Nitrilases catalyze the conversion of organic nitriles to corresponding acids and ammonia, while nitrile hydratases (NHase) catalyze the formation of amides from nitriles, which is subsequently converted to acids and ammonia by amidases [Nagasawa et al, 1988]. Hydrogen cyanide abiotically formed from various metal cyanides leads to various products by different enzymes, such as, to CO_2 and NH_3 by cyanase [Dorr et al, 1989], to formamide by cyanide hydratase [Wang et al, 1992], or to formic acid and ammonia by cyanide dihydratase [Ingovorsen et al, 1991]. Many plants and insects oxidize some of the nitriles to cyanohydrins (α-hydroxynitriles) by oxygenases, which are further converted to an aldehyde and hydrogen cyanide by oxynitrilases (hydroxynitrile lyases) [Johnson et al, 2000]. This type of enzymatic system is almost nonexistent in microorganisms. However, a *Trichoderma* sp. was reported to degrade diaminomalenonitrile, releasing HCN [Kuwahara et al, 1985]. Nitrogenase, present in the nitrogen-fixing organisms is capable of reducing atmospheric nitrogen and nitrile compounds to corresponding hydrocarbon and ammonia [Liu et al, 1997].

1.3.1.1. Nitrilases

The enzyme nitrilase was first described by Thimann and Mahadevan in1964 [Thimann, 1964 #59]. The enzyme, which was isolated from barley leaves, catalysed the conversion of indoleacetonitrile (IAN) to indoleacetic acid (IAA) and was initially called indoleacetonitrilase. Substrate analysis with purified enzyme on 26 nitriles indicated that the enzyme had a broad substrate range. The rate of hydrolysis was eight times greater with 3-cyanopyridine than with IAN. The enzyme was therefore renamed nitrilase to indicate the broad substrate range of the enzyme [Thimann et al, 1964]. The first bacterial nitrilase was isolated from a soil bacterium (possibly a *Pseudomonas* species) by selection for growth on the naturally occurring nitrile, ricinine (N-methyl-3-cyano-4-methoxy-2-pyridone), as a sole carbon source [Hook et al, 1964]. This enzyme catalysed the hydrolysis of a range of 2-pyridones with rates between 0 and 118% relative to ricinine (100%).

The biotechnological potential of nitrile hydrolysing enzymes has led to the isolation of a range of bacteria and fungi capable of hydrolysing nitriles [Kobayashi, 1997 #218], even from extremophiles [Cowan et al, 1998]. Most of these were isolated on the basis of using a particular nitrile as a carbon and/or nitriogen source. A list of bacteria, fungi and plants with well-characterized nitrilases is given in Table 1. The enzymes studied to date show a very diverse range of biochemical characteristics. In particular substrate specificity of the enzymes varies widely. Initial investigations suggested that nitrilases were specific for aromatic nitriles and nitrile hydratases for aliphatic nitriles but this distinction has to be reconsidered in the light of the growing body of information on both nitrilases and nitrile hydratases. None of the enzymes show identical properties. The most notable differences between the enzymes are in substrate specificity, native structure and aggregation properties, and pH optima.

1.3.1.2. Distribution and physiological role of nitrilases

Nitrilases are found relatively infrequently in nature. The existence of the enzyme activity in 3 out of 21 plant families (*Gramineae*, *Cruciferae*, and *Musaceae*) [Thimann et al, 1964] and in a limited number of fungal genera (*Fusarium*, *Aspergillus*, *Penicillium*) [Harper et al, 1977a] indicates the relative rarity of this activity. Nitrile-degrading activity is more frequent in bacteria, though without extensive screening it is almost impossible to assess the actual distribution frequency. A number of bacteria (*Acinetobacter*, *Corynebacterium*, *Arthrobacter*, *Pseudomonas, Klebsiella*, *Nocardia*, *Rhodococcus*, etc.) are known to utilize nitriles as sole source of carbon and nitrogen. The physiological role of nitrile hydrolysing enzymes in microorganisms is not clear. In plants, such activities are implicated in nutrient metabolism, particularly in the degradation of glucosinolates [Bestwick et al, 1993] and in the synthesis of indole acetic acid [Bartel et al, 1994]. The genome sequence of *Arabidopsis thaliana* revealed four nitrilase related sequences (AtNIT 1, 2, 3 and 4) [Bartling et al, 1992]. AtNIT1, 2 and 3 are isoenzymes, which are found only in *Brassicaceae* [Hillebrand et al, 1998]. AtNIT4 on the other hand is not linked to the other three nitrilases and its homologs are found in many plant species such as tobacco [Tsunoda et al, 1995]

and rice [Piotrowski et al, 2001]. Though indole 3-acetonitrile is a relatively poor substrate for AtNIT1-3 enzymes, but it is postulated to have a role in auxin biosynthesis under certain circumstances [Vorwerk et al, 2001]. The major physiological role for these enzymes appears to be glucosinolate metabolism. AtNIT4 is a β-cyano alanine hydratase and plays a role in cyanide detoxification [Piotrowski et al, 2001]. In microbial system nitrilases probably form components of complex pathway controlling production and degradation of aldoximes, which are key intermediates. Nitriles, which are formed by upstream enzyme activities of aldoxime dehydratases may further undergo hydrolysis, oxidation, reduction etc. by different enzymes including nitrilases (Figure 2).

1.3.1.3. Enzyme structure

The subunit molecular mass and native structure has been determined for many nitrilases. Most nitrilases consist of a single polypeptide with a molecular mass of approximately 40 kDa (32–45 kDa), which aggregates to form the active enzyme. The preferred form of the enzyme seems to be a large aggregate of 6–26 subunits.

Table 1. Characteristics of some purified nitrilases

Organism	Substrate	Molecular mass (kDa)		pH optima	Temperature optima (°C)	Reference
		Native	Subunit			
Acinebacter sp. AK226	Ibu-CN 100% Acrylonitrile 144% Benzonitrile 94%	580	43	8	50	[Yamamoto, 1991]
Alcaligenes faecalis ATCC 8750	Mandelonitrile 100% p-aminobenzyl cyanide 1670% Benzonitrile 1.1%	460	32	7.5	40–45	[Yamamoto, 1992]
Alcaligenes faecalis JM3	Mandelonitrile 8.62% p-aminobenzyl cyanide 49.1% 2-thiophenacetonitrile 100%	260	44	7.5	45	[Nagasawa, 1990] [Kobayashi, 1993]
Bacillus pallidus Dac521	Benzonitrile 100% Crotononitrile 80.3% Acrylonitrile 8.9%	600	41 + 72 (groEL)	6–9	65	[Almatawah, 1999]
Comamonas testosteroni	Adiponitrile 100% Acrylonitrile 23% Benzonitrile 4%	Oligomer	38			[Schill, 1995]
Fusarium oxysporum f.sp. melonis	Benzonitrile 100% Acrylonitrile 35% Crotononitrile 17%	550 (170–880)	37	6–11	40	[Goldhust, 1989]
Fusarium solani IMI196840	Benzonitrile 100% Acetonitrile 6.6% 1,4-Benzodinitrile 213.3%	620	76	7.8–9.1		[Harper, 1977a]
Klebsiella pneumoniae ssp. ozaenae	Bromoxynil 100% Benzonitrile <0.005%	74	37	9.2	35	[Stalker, 1988]
Pseudomonas sp. (SI)	Acrylonitrile 100% Acetonitrile 31.5% Benzonitrile 3.8%	41	41			[Dhillon, 1999]
Nocardia (*Rhodococcus*) NCIB11216	Benzonitrile 100% m-bromobenzonitrile 174.8%	560	45 Substrate activation	8	30	[Harper, 1977b] [Hoyle, 1998]
Nocardia (*Rhodococcus*) NCIB11215	Benzonitrile 100% m-bromobenzonitrile 58.4% m-nitrobenzonitrile 841.7	560	45	7–9.5	30	[Harper, 1985]
Rhodococcus ATCC39484	Benzonitrile 100% 2-furanocarbonitrile 171%	560	40 Substrate activation	7.5	30	[Stevenson, 1992]
Rhodococcus rhodococcus K22	Crotononitrile 100% Acrylonitrile 348% Benzonitrile 27.1%	650	41	5.5	55	[Kobayashi, 1990]
Rhodococcus rhodochrous PA-34	Benzonitrile 100% Acrylonitrile 22.4 Crotononitrile 20.9%	45	45	7.5	35	[Bhalla, 1992]
Rhodococcus rhodochrous J1	Benzonitrile 100% Acrylonitrile 127% Crotononitrile 27%	410	40 Substrate activation	7.6	45	[Kobayashi, 1989] [Nagasawa, 2000]

The enzyme of *R. rhodochrous* PA-34 is active as a monomer with a molecular mass of 45 kDa [Bhalla et al, 1992]. The bromoxynil-specific nitrilase of *Klebsiella pneumoniae* ssp. ozaenae is active as a dimmer [Stalker et al, 1988]. The nitrilase of *Fusarium solani* IMI196840 is unusual, in that it has a subunit molecular mass of 72 kDa. Other characteristics of this enzyme are similar to other nitrilases and in particular the enzyme aggregates to an active form of 620 kDa [Harper et al, 1977a]. Some of these enzymes aggregate as a consequence of substrate activation. This phenomenon was first observed with the enzyme from *Nocardia* (*Rhodococcus*) NCIB 11216 [Harper et al, 1977b]. Nitrilase purified in the absence of benzonitrile had an elution volume, on gel filtration, that corresponded to a molecular mass of 47 kDa while, for the enzyme purified in the presence of benzonitrile, molecular mass was determined to be 560 kDa. The association of the subunits was shown to be pH, temperature and enzyme concentration dependent with a pH optimum of 7.3 and very slow association below 20°C. The nitrilases of *Rhodococcus* ATCC 39484, *Alcaligenes faecalis* ATCC8750 and *R. rhodochrous* J1 also show substrate-dependent activation [Stevenson et al, 1992; Yamamoto et al, 1992; Nagasawa et al, 2000]. It was also demonstrated that subunit association of the nitrilase of *R. rhodochrous* J1 took place in the presence of benzonitrile but not in the presence of acrylonitrile although acrylonitrile is a good substrate for the activated enzyme [Nagasawa et al, 2000]. In addition to substrate dependent activation most of the enzymes also show similar phenomenon in the presence of higher concentration of salt, organic solvent, pH, temperature and enzyme concentration may also trigger the subunit association and therefore activation [Nagasawa et al, 2000]. The hydrophobic effect resulting from the presence of the above mentioned conditions might change the conformation of the enzyme exhibiting hydrophobic sites and thereby enabling subunit assembly and enzyme activation. The nitrilases of *Pseudomonas fluorescens* DSM 7155 and *Bacillus pallidus* Dac 521 co-purify with chaperonins [Layh et al, 1998a, Almatawah et al, 1999]. It was suggested that chaperonins might play role in the assembly of the subunits into high molecular weight complex and also stabilizing the complex. When the nitrilase

gene of *Comamonas testosteroni* was cloned and over-expressed in *E. coli*, as high as 30% of the total cellular protein was found to be nitrilase, but only 10% of this was soluble in nature. The solubility of nitrilase was enhanced by co-overexpression of GroEL (chaperonin) in the same cell and the activity increased five fold [Schill et al, 1995]. This further confirms the crucial role of chaperonins in the correct folding and subunit association of nitrilase.

1.3.1.4. Reaction mechanism

Hydrolysis of a nitrile of the form R-C≡N produces the corresponding amide, R-C=O(NH_2), with the addition of one molecule of water and the corresponding acid, R-CO_2H, with the addition of second molecule of water. Nitrilases are interesting enzyme in the sense that the substrates are nitriles but the reaction does not involve release of, or reaction with a substantial amount of the corresponding amide. However, it has been shown that the purified nitrilases of *Fusarium oxysporum* ssp. *Melonis* [Goldhust et al, 1989], *R. rhodochrous* ATCC 39484 [Stevenson et al, 1992], *Pseudomonas fluorescens* DSM7155 [Layh, 1998a] and the ricinine nitrilase of *Pseuodomonas* sp. [Hook, 1964] produce a small amount of amide product and therefore have nitrile hydratase activity. In these cases the amide product is usually <5% of the total reaction products. AtNIT1 and AtNIT4 also have NHase activity, especially AtNIT4, which produces 1.5 times more asparagine than aspartic acid from β-cyano alanine [Piotrowski et al, 2001]. Some of the nitrilases use amides, albeit at a very low rate compared to nitrile substrate and therefore has amidase activity [Kobayashi et al, 1998]. A reaction mechanism was proposed which accounts for all these activities (Figure 3). The mechanism which is in accordance with the earlier mechanism [Hook et al, 1964; Thimann et al, 1964], involves nucleophilic attack on the nitrile carbon by sulfhydryl group of the nitrilase, leading to a tetrahedral intermediate via an enzyme thioimidate route. Often the tetrahedral intermediate can break down anomalously to produce amide instead of normal acid product. The geometric constraints of this reaction suggest that nitrilase facilitates interaction with a

linear (approximately 180°) substrate, planar (approximately 120°) thioimidate and acylenzyme intermediates, and tetrahedral (approximately 109.5°) water-bonded intermediates. Because ammonia is a better leaving group than the enzyme, the first enzyme-dependent water addition does not produce an acid amide but rather the acylenzyme complex, hydrolysis of which produces the acid product. Most nitrilases bind strongly to a bulky substrate R group in a conformation that places the second carbon closer to 120° than to 180° from the cyano nitrogen. Fitting a distorted substrate nitrile would push the substrate towards thioimidation and would reduce the geometric sweeps required of enzyme complexes. In support of this view, most nitrilases prefer bulky substrates to non-substituted acetonitrile [Pace et al, 2001].

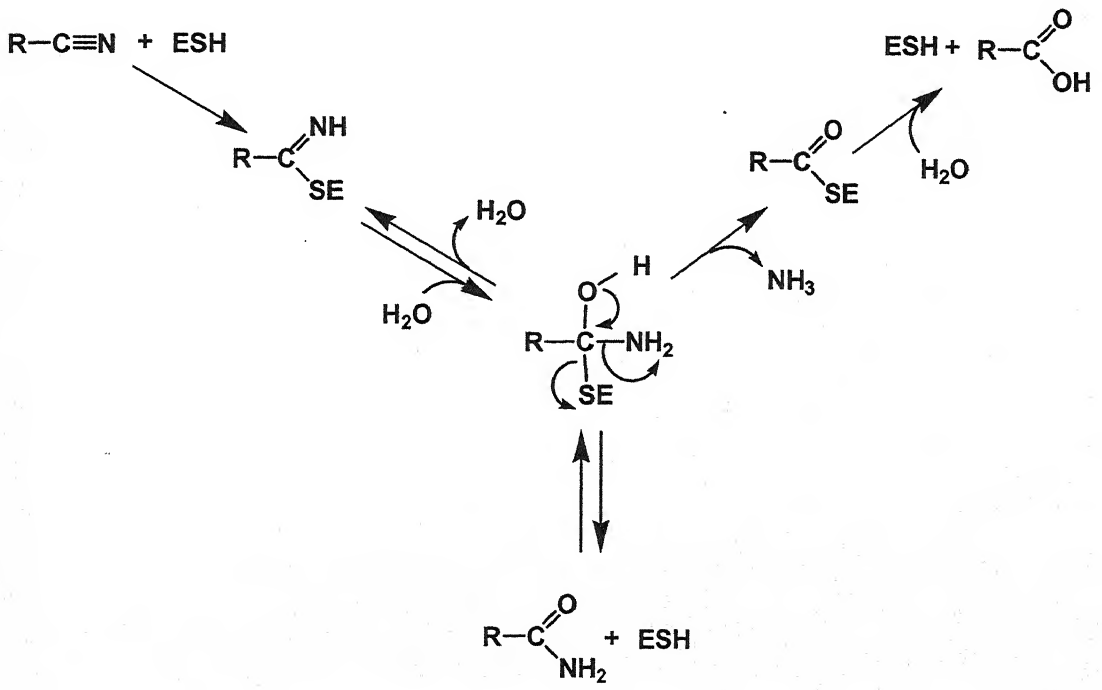


Figure 5. Reaction mechanism of nitrilase catalysis

1.3.2. Applications

Biotransformation of nitriles provides great potential for synthetic chemists. The ability of the enzyme system to convert cyano functionality to either an acid or an amide is, in itself, of great use. Traditional chemical methods for conversion of nitriles to acids or amides have several drawbacks: (1) reactions need to be carried out either in strongly acidic (6 M HCl) or basic conditions (2 M NaOH) under constant reflux [Vogel et al, 1989], (2) higher reaction temperature, (3) formation of by-products such as toxic HCN or large amounts of salts, etc. Biocatalytic hydrolysis of nitriles is attractive due to its ability to effect reactions in a more-green manner and because of the potential for carrying out *chemo*-, *regio*-, and *enantio*-selective transformations.

1.3.2.1. Bioremediation

Synthetic nitrile compounds are widespread in the environment in the form of industrial wastewater. Most of these are toxic, carcinogenic and mutagenic in nature [Pollak et al, 1991] and thus there is a need to control their release into the environment. A mixed culture of bacteria containing different nitrile hydrolyzing enzymes (including NHase, amidase and nitrilase) that metabolise effluent containing acrylonitrile, fumaronitrile, succinonitrile, etc. were grown in batch and continuous culture on these components of waste [Wyatt et al, 1995]. A reduction in chemical oxygen demand (75%) and significant removal (99%) of detectable toxic components was achieved by biodegradation of the effluent from acrylonitrile manufacturing industries using mixed cultures of bacteria [Wyatt et al, 1995]. The use of specialized consortia of microorganisms to degrade toxic wastes therefore could be a viable alternative approach to the classical activated sludge system.

Prolonged exposure to nitrile herbicides [dichlorobenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-dibromo-4- hydroxybenzonitrile) etc.] results in symptoms of weight loss, fever, vomiting, headache and urinary problems [Freyssinet et al, 1996]. Nitrile-metabolizing enzymes efficiently degrade these cyano group-

containing herbicides and prevent them from entering the food chain. *Agrobacterium radiobacter*, a bromoxynil-degrading soil bacterium, was used for the degradation of the herbicide under nonsterile batch and continuous conditions with 65% reduction in the bromoxynil concentration in a column reactor after 5 days. The efficacy of degradation is enhanced by the addition of ferrous, cobaltous or cupric ions [Muller et al, 1999]. A gene encoding the nitrilase has been cloned from *Klebsiella pneumoniae* ssp. *ozaenae* [McBride et al, 1986] and used to raise herbicide resistant plants [Stalker et al, 1988]. Bromoxynil resistant transgenic plants resulting from the introduction of microbial bromoxynil specific nitrilase genes into tomato or tobacco are already approved for commercial use [Freyssinet et al, 1996]. Similarly, other nitrile-degrading enzymes could also be potential candidates for molecular manipulation of bio-degradative systems in plant biotechnology.

1.3.2.2. Synthetic biocatalysis

Industrial scale processes for the production of acrylamide (Mitsubishi Rayon Co. Japan), [Nagasawa et al, 1993; Yamada et al, 1994] and nicotinamide (Lonza AG, Switzerland) [Chassin et al 1996] involving nitrile converting enzymes are the two most prominent examples of biocatalytic reaction being implemented at higher scale and pave the path for the development of other processes involving this class of enzymes. Nitrilases can also selectively convert one cyano group of a polynitrile, which is virtually impossible using conventional chemical methods. *R. rhodochrous* K22 catalyzes the conversion of adiponitrile to 5-cyanovaleric acid, which is an intermediate for the synthesis of nylon-6 [Godtfredsen et al 1985] and Tranexamic acid, a homeostatic drug, is obtained by selective mono-hydrolysis of *trans* 1,4-dicyano cyclohexane by *Acremonium* sp. [Nishise et al, 1987]. Nitrilases also offer the possibility of stereoselective transformation which includes the production of several important fine chemicals and pharmaceutical intermediates, for example (*S*)-phenyllactic acid [Hashimoto et al, 1996], L-α-amino acids [Bhalla et al, 1992], (*R*)-β-hydroxy acids [Wu et al, 2003], (*S*)-

ibuprofen [Yamamoto et al 1990] etc. Although in recent years several applications of nitrilases have been recognized [Kobayashi et al, 1994; Sugai et al, 1997] to date there exists only two industrial scale processes for the production of 1,5-dimethyl 2-piperidone (DuPont, USA) and (*R*)-(-)-mandelic acid (BASF, Germany; Mitsubishi Rayon, Japan) employing these as biocatalysts. 1,5-dimethyl 2-piperidone (Xolvone^{TM}) which has desirable properties for electronics, coatings and solvent applications, is produced from 4-cyanopentanoic acid, which in turn is generated from *regio*-selective nitrilase mediated transformation of 2-methyl glutaronitrile (MGN) employing *Acidovorax facilis* 72W cells [Gavagan et al, 1998; DiCosimo et al, 2000; Cooling et al, 2001]. When compared to existing chemical process in which methylglutaronitrile is directly converted via hydrogenation to a mixture of 1,3-dimethyl-2-piperidone and 1,5-dimethyl-2-piperidone, the chemoenzymatic method generates less waste and produces a single lactam isomer in significantly higher yield. BASF and Mitsubishi Rayon use nitrilase mediated processes for the production of (*R*)-(-)-mandelic acid and its derivatives on a multiton scale [Endo et al, 1993; Ress-Loschke et al, 1998]. The advantage of this process over its chemical counterpart is that no organic solvent is required and high degree of stereoselectivity being achieved because of the presence of the biocatalyst.

1.3.3. Research interest in mandelic acid

Mandelic acid, a α-hydroxy acid named after the German *mandel* (almond), is derived from the hydrolysis of bitter almond extracts. The research interest in mandelic acid stems from its dual nature, as an important pharmaceutical intermediate and a specialty chemical. (*R*)-(-)-mandelic acid is an useful resolving agent [Kinbara et al, 1996; Drabowics et al, 1999; Han et al, 2002] and a chiral synthon for the production of various pharmaceuticals, such as, semisynthetic penicillins [Furlenmeier et al, 1976], cephalosporins [Terreni et al, 2001], anti-obesity agents [Mills et al, 1983], antiarrhythmic agents [Aav, et al 1999], antitumor agents [Kotora, 1996; Surivet, 1996; Surivet, 1997; Surivet,

1998; Surivet et al, 1999] and it exhibits antifungal properties [Kope et al, 1991]. Mandelic acid has also been used as a broad-spectrum β-lactamase inhibitor [Mollard et al, 2001], antithrombotic agent [Su et al, 2001], antioxidative agent [Ley, 2001] and to prevent abnormal skin pigmentation [Ley et al, 2001]. It has been used in medicine for many years as urinary antiseptic. Methenamine mandelate (Mandelamine®, Parke-Davis), a combination of methenamine and mandelic acid, is used as a urinary antiseptic and inhibits *Staphylococcus aureus, Bacillus proteus, E. coli* and *Aerobacter aerogenes* at a concentration of 35 to 50 g/100 L [Fuursted et al, 1997]. Mandelic acid products, used alone or in tandem with antioxidant vitamins, have multiple beneficial effects as cosmeceutical agent, which includes antibacterial effect and improvement in photo-aged skin, acne, abnormal pigmentation, and skin texture. Chemical peeling with mandelic acid, when compared with glycolic acid, produces less erythema, and is less likely to result in crusting or blistering or other adverse effects on the epidermis. Melasma, postinflammatory hyperpigmentation and lentigines improved quickly when treated with mandelic acid products [Taylor et al, 1999]. Recently, a mandelic acid condensation polymer has proven to be a novel antiviral agent against human immunodeficiency virus and herpes simplex virus and a new antimicrobial contraceptive for vaginal prophylaxis [Herold et al, 2002].

1.3.4. Routes to optically pure mandelic acid

Conventionally optically pure mandelic acid is produced by optical resolution of the racemate with chiral amines. The method is not economical enough due to the use of expensive chiral resolving agents. It can also be produced enzymatically via lipases [Kimura et al, 2002], esterases [Mori et al, 1980], glyoxylases [Patterson et al, 1981], mandelate dehydrogenases [Yamazaki et al, 1986], and nitrilases [Yamamoto et al, 1991] [Rustler et al, 2007; Rey et al, 2004]. Using benzaldehyde and hydrogen cyanide as a cheap starting material, enantioselective cyanation was performed using hydroxynitrile lyase and the

resulting (*R*)-mandelonitrile was hydrolysed to yiled (*R*)-mandelic acid employing a nitrilase [Mateo et al, 2006]. Using a bienzymatic scheme employing a lipase and a mandelate racemase, preparation of (*S*)-enantiomer of the acid has also been reported [Strauss et al, 1999]. Majority of these enzymatic methods suffer from several drawbacks, which include use of expensive co-factors and operational stability of the enzyme concerned.

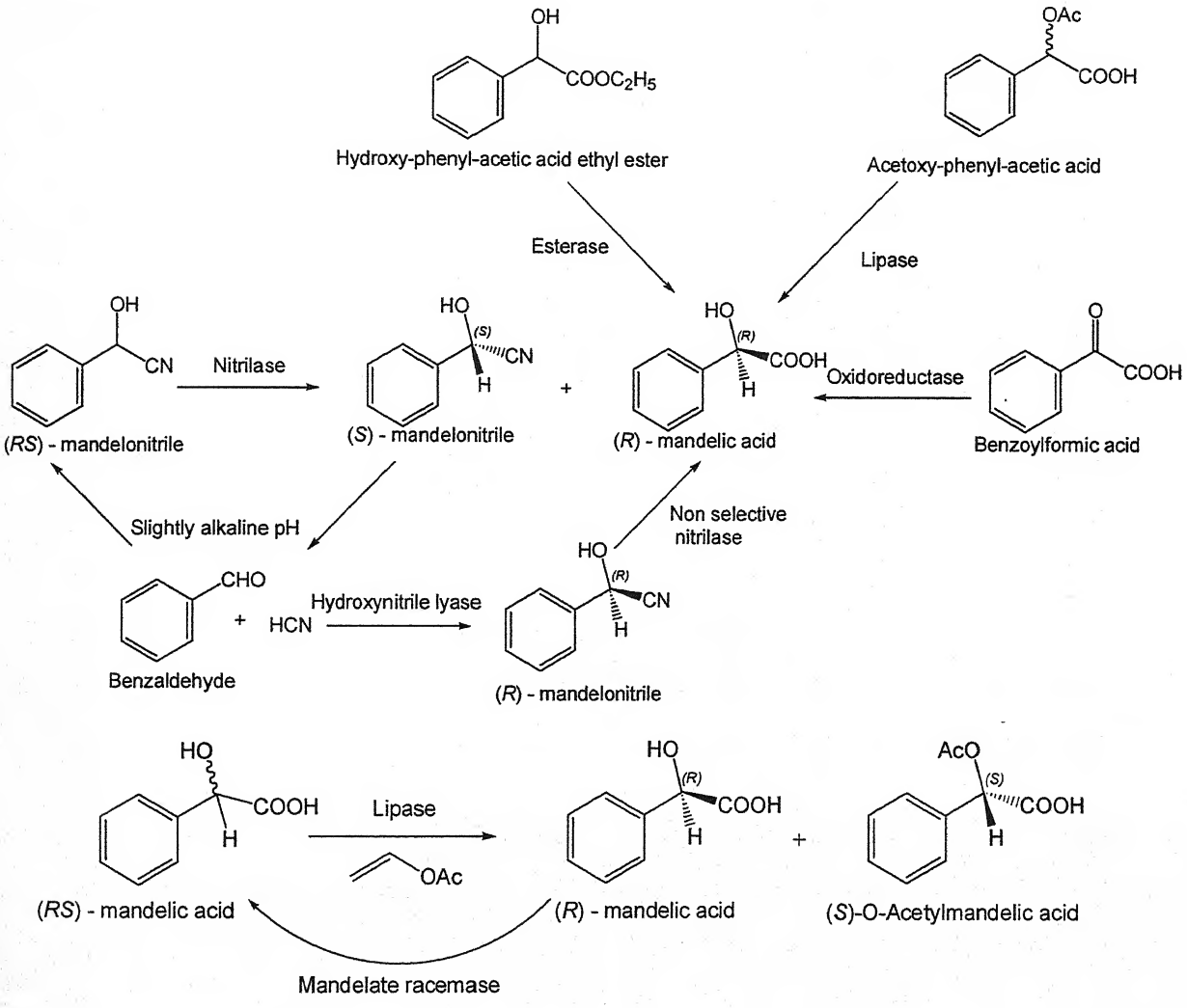


Figure 6. Various schemes for enzymatic preparation of optically pure mandelic acid

The pathways involving lipases and esterases are kinetic resolution process in which at the most 50% yield of the desired product can be expected and there is also the involvement of an additional step in separating the non-reactive isomer, which increases the cost of overall process. The oxidoreductase pathway is quite promising, as it offers 100% theoretical yield because of asymmetric reduction, but requires costly co-factors. Although in current date there are several co-factor regeneration strategies available, applications at the higher scale have been limited. A bi-enzymatic pathway involving lipase and mandelate racemase can also give rise to 100% yield with high enantiomeric excess of the desired product. However, the pathways involving racemases or lyases are not that well explored and sources of this class of enzymes are also limited. Moreover, all these processes involve use of expensive substrates, which are not readily available.

Nitrilase mediated pathway on the other hand has edge over other procedures as it does not involve expensive co-factors and the substrate mandelonitrile is readily available and cheaper. Nitrilase mediated hydrolysis appears to be a classical kinetic resolution giving a theoretical maximum yield of 50%. Under kinetic conditions, the unreacted (*S*)-substrate enantiomer complicates its separation from the product of interest. However, by adjusting the pH to slightly alkaline, the (*S*)-mandelonitrile can be funneled back into the racemization pathway. Thus, at alkaline pH dynamic conditions prevail, where kinetic resolution is coupled to racemization so that the chirally labile substrate continuously equilibrates to give 100% single enantiomer of the product. Yamamoto *et al.* (1991) first reported (*R*)-(-)-mandelic acid production in high yield from racemic mandelonitrile employing an *R*-selective nitrilase from *A. faecalis* ATCC 8750 [Yamamoto et al, 1991]. Later on BASF (Germany) and Mitshubishi Rayon Co. (Japan) exploited the potential of nitrilases and has recently come up with multiton scale processes for (*R*)-(-)-mandelic acid employing nitrilases [Endo et al, 1993, Ress-Loschke et al, 1998]. In an effort to harness the most diversified range of enzymes that can be found in nature, Diversa Corporation (California, USA) have created large genomic libraries by extracting DNA directly from environmental samples from various habitats, and

identified 200 new nitrilases [Robertson et al, 2004], 27 of which afforded (*R*)-(-)-mandelic acid with >90% enantiomeric excess [DeSantis et al, 2002]. Diversa has also identified and created a variant of the nitrilase by a novel directed evolution technique (Gene site saturation mutagenesis) that could effect selective and mild hydrolysis of 3-hydroxy glutaronitrile to afford (*R*)-4-cyano-3-hydroxy butyric acid, the ethyl ester of which is an intermediate of the cholesterol lowering blockbuster drug Lipitor [DeSantis et al, 2003]. Extensive worldwide research is being carried out especially in different industries like Dupont, Lonza, Dow, Diversa, DSM, BASF etc., to harness the potential of this very promising enzyme.

1.3.5. Dilemma with nitrile biotransformation

Inspite of the great synthetic potential of nitrilases for the production of high-value optically pure carboxylic acids [Singh et al, 2006; Martínková et al, 2002], their utilization as a versatile biocatalyst is largely unexploited, considering the successful application of lipases and esterases in enantioselective synthesis. The major reason that has weighed down their synthetic application is their highly unstable nature and availability in a cheap, reusable and stable preparation. A wider applicability of nitrilase mediated biotransformation would be promoted by a greater availability of commercial nitrilases in a stabilized formulation. One of the bottlenecks in development of a specific biotransformation is to find the appropriate biocatalyst [Burton et al, 2002]. Moreover, insolubility of the nitrile substrate in aqueous reaction mixture presents an additional challenge to enzymatic nitrile hydrolysis. In this regard, the use of organic solvents to enhance the availability of insoluble nitrile substrate to nitrilase active site has been a very restricted approach [Layh et al, 1988b], since its behavior in such non conventional media is not fully understood. Moreover, solvent engineering has been regarded as an appealing alternative to protein engineering [Klibanov et al, 2001].

1.3.6. Significance of the Work

The importance of nitrilase in the transformation of nitrile compounds to corresponding acids, Study the enantioselective hydrolysis of racemic mandelonitrile to (*R*)-(-)-mandelic acid. Since, (*R*)-(-)-mandelic acid is an important fine chemical, production of (*R*)-(-)-mandelic acid with high enantiomeric excess at high volumetric productivities from readily available mandelonitrile employing nitrilases. Nitrilases are not cheaply available in market and currently limited in variety and catalytic applications. Development of cheap nutrient medium with the replacement of IPTG by lactose. This has opened up a fascinating opportunity to develop a cheap nutrient medium devoid of IPTG for the growth of the recombinant cells, the cost of the medium will be substantially low and economic feasibility of the process will be higher. Biocatalytic transformation of mandelonitrile provides significant advantages over other alternative routes to produce enantiopure mandelic acid. Broaden the applicability of whole cell biocatalyst, Entrapment of whole cells of recombinant *E. coli* affect the initial reaction rate, conversion and selectivity of the biotransformation and at the same time stabilize the biocatalyst to allow its efficient reusability

2. OBJECTIVE

Keeping in view the importance of nitrilase in the transformation of nitrile compounds to corresponding acids, realizing the importance of growth and production of nitrilase by the recombinant organism (*E. coli*) in a stirred tank reactor and immobtilization of whole cell biocatalysis, it is desirable to study stereoselective hydrolysis of mandelonitrile to (*R*)-(-)- mandelic acid. The objective of the proposed project was to optimize the scale up for the production of a nitrile hydrolysing biocatalyst from *E. coli* and then to optimize the reaction conditions for carrying out the desired reaction.

The following were the specific aims:

1. Optimization of media composition for the growth and nitrilase production by *E. coli.*
2. Study the effect of following parameters on the growth and nitrilase production *E. coli.*
3. Determination of mass transfer coefficient in the stirred tank reactor
4. Entrapment of Whole cells of recombinant E.coli and chaeracterization of most suitable biocatalyst support
5. Optimization of various physico-chemical parameters for the biotransformation of mandelonitrile to mandelic acid

Materials & Methods

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Mandelonitrile was obtained from Aldrich chemical company (Milawukee, U.S.A.). All media components were supplied by Hi Media laboratories Ltd. (Mumbai, India). All inorganic salts were supplied by Qualigens Fine Chemicals (Mumbal, India). All other reagents or chemicals used were of analytical grade and obtained from standard companies.

3.1.2. Microorganism and cultivation condition

Nitrilase gene from *Pseudomonas putida* MTCC 5110 was cloned in *E. coli* BL21 (DE3) in our laboratory and that was used for the studies. The stock culture was maintained on Luria Bertani plates containing ampicillin. The microorganism was initially grown at 37°C for 16 h in a medium of the following composition - yeast extract (10 g/l), tryptone (16 g/l) and sodium chloride (5 g/l). After 16 h, 10% (v/v) of the culture was transferred to flasks containing of the same. Lactose was added after 1 h of transferring as an inducer at a concentration of 0.8%.

3.1.3. Bioreactor and accessories

The studies were carried out in a 2.5 l benchtop fermenter (BIOFLO 3000, New Brunswick Scientific Co., NJ, USA) having a working volume of 2 l. The vessel has a flanged glass tube body detachable from stainless steel head plate and stainless steel bottom dished head. The bottom is jacketed for circulation of temperature-controlled water. Ports are present in the headplate for inoculation and acid-base addition. The headplate also has arrangements for a foam probe, sparger, harvesting tube, exhaust condenser, pH and dissolved oxygen electrodes, and a thermowell for temperature sensor. The agitation system consists of a removable motor located on top of the vessel and connected to the agitation shaft with a multi jaw coupling. The agitation shaft is made of stainless steel and has two six-blade stainless steel impellers.. The temperature was

controlled by circulating chilled water from a chilling water bath (Julabo, Germany).

3.2. Methods

3.2.1. Analytical methods

3.2.1.1 Estimation of nitrilase activity

Nitrilase activity of the microorganisms was determined either by estimating the liberated ammonia or by measuring the amount of carboxylic acid that was formed in the reaction mixture due to nitrile hydrolysis. Enzyme activity was determined in reaction mixture containing 0.1 M phosphate buffer (pH 7.5), 50 µl 200 mM mendelonitrle and 200 µl 50 mg/ml cell suspension. The reaction mixture was incubated at 37°C for 20 min and 200 µl 1N HCl was added to stop the reaction. Finally, the reaction mixture was centrifuged (Sigma 6K15, GmbH, Germany) at 10,000 x g to remove the cells or to precipitate the protein. Clear supernatant thus obtained was used for the estimation of liberated ammonia by Berthelot method. One unit of nitrile hydrolyzing activity is defined as the amount of enzyme capable of producing 1 µmol of ammonia or mandelic acid under standard assay condition.

3.1.1.2 Berthelot method

The liberated ammonia from the nitrilase catalyzed reaction was reacted with phenol and sodium nitroprusside in presence of sodium hypochlorite to produce indophenol derivative which produced a blue chromophore (λmax 640 nm).

Reagents used

Solution A: Phenol 10 g and sodium nitroprusside 0.05 g were dissolved in 1 L distilled water to obtain solution A.

Solution B: Sodium hydroxide 5 g was dissolved in 1 L distilled water and 5 ml sodium hypochlorite was added to the solution which was designated as solution B.

Procedure

To 1 ml reaction supernatant, 2.5 ml solution A was added and thoroughly mixed. After incubation for 5 min at room temperature, 2.5 ml solution B was added and the chromophore was allowed to develop by incubating the reaction mixture in boiling water bath for 5 min. The absorbance of the resulting chromophore was recorded at 640 nm against control sample using UV-Vis Spectrophotometer (Beckman DU 7400, Fullerton, USA).

3.2.2 Cell mass estimation

Culture broth (1 ml) was centrifuged at 10, 000 x g for 10 min and the cell pellet thus obtained was washed with phosphate buffer (0.1 M, pH 7.5). Finally, the cell pellet was resuspended in 1 ml buffer and absorbance was measured at 600 nm in an UV-Vis Spectrophotometer (Beckman DU 7400, Fullerton, USA).

3.2.3 Media optimization

3.2.3.1 Effect of carbon source

Various carbon sources like glucose, fructose, sucrose, lactose, glycerol, starch, sorbitol, succinate and citrate were used at a final concentration of 0.8 g/l. The microorganism was grown aerobically in an orbital shaker (200 rpm) at 37°C, pH 7.5 and cell mass and nitrilase activity were estimated.

3.2.3.2. Effect of nitrogen source

The effect of different organic and inorganic nitrogen sources such as, peptone, tryptone, ammonium chloride, ammonium nitrate and urea were examined on the growth and nitrilase production by *E. coli* at a final concentration of 16 g/l.

3.2.3.3. Effect of growth factor

Different growth factors like beef extract, yeast extract, brain heart infusion, malt extract and meat extract (final concentration 10 g/l) were investigated for their ability to induce maximum cell mass and enzyme production.

3.2.4. Bioreactor studies

3.2.4.1. Cultivation at different initial pH in the bioreactor

After optimization studies at shake flask level, experiments determining the effect of different physicochemical parameters on the growth and enzyme production

were carried out in a laboratory scale fermenter. The medium was prepared and the initial pH was adjusted to 6.5, 7.5 and 8.5 while the agitation and temperature were set at 200 rpm and 37°C respectively. The bioreactor was run with these parameters and the pH changes were monitored. Enzyme activity, cell mass and residual sugar (total) concentration was determined at regular time intervals.

3.2.4.2. Cultivation at different agitation rates in the bioreactor

To determine the optimum agitation speed in the bioreactor, studies were carried out at different agitation rates of 200, 300 and 400 rpm. The initial pH for these set of experiments was set at 7.5 and the temperature at 37°C. Cell mass and residual total sugar concentrations were determined periodically.

3.2.4.3. Cultivation at different aeration rates in the bioreactor

In aerobic process, oxygen is an important co-substrate. Oxygen must be continuously supplied to the process for acceptable productivities and high growth to be achieved. At the same time its solubility is quite low in aqueous solution while the demand is quite high. Hence studies to enhance the efficiency of oxygen mass transfer by increasing aeration rate were carried out. Consequently different aeration rates of 0.5, 1 and 1.5 were used.

3.2.4.4. Cultivation at different controlled pH in the bioreactor

In these set of experiments the pH of the fermentation media was controlled at a particular level throughout the experiment. This was achieved by the addition of 6N H_2SO_4 / 10N NaOH intermittently as and when required. The pH was kept constant at 6.5, 7.5 and 8.5 during which the enzyme activity, growth and starch concentration were determined periodically.

3.2.5. Determination of the volumetric oxygen transfer coefficient (KLa) and effect of different parameters on KLa

The volumetric mass transfer coefficient (KLa) is used to measure the aeration efficiency of the fermenter. Although the literature cites different methods of KLa determination, the most widely used include the gassing out techniques, i.e. dynamic and static gassing out methods. Static gassing out method Static gassing-out method (Atkinson and Mavituna, 1991) describes the determination

of KLa for non-respiring system. In this technique the oxygen transfer coefficient was determined by scrubbing the media free of oxygen by passing non oxygen containing gas (e.g. nitrogen) through it. Air was then passed through the media and the rise of the dissolved oxygen concentration is monitored. The increase in the dissolved oxygen (DO) concentration is represented as

$$dCL/dt = KLa(C^* - CL) \quad (1)$$

Where

dCL/dt = oxygen transfer rate (g/l.h)

KL = mass transfer coefficient (h-1)

a = interface area (m)

C* = saturated oxygen concentration (g/l)

CL = concentration of dissolved oxygen (g/l)

t = time (h)

Integrating equation (1), we have

$$\ln [1-(CL/ C^*)] = - KLa . t \quad (2)$$

A plot of ln [1-(CL/ C*)] with time yields a straight line, the negative slope of which Corresponds to the volumetric mass transfer coefficient.

3.2.5.1. Dynamic gassing out method

This method is used to determine the KLa of a respiring system. Therefore the method was utilized during the fermentation process, thus giving the benefit of being more realistic as compared to static gassing out method. The procedure involved stopping the oxygen supply to the bioreactor during the course of the fermentation process. As a result the dissolved oxygen level in the system goes on decreasing due to oxygen consumption by microorganisms. At a certain point the aeration was resumed and the DO concentration allowed increasing to its earlier level. The observed increase in the DO concentration is the difference between the transfer of oxygen into solution and its uptake by the respiring organisms. This uptake is expressed by equation (3)

$dCL/dt = KLa(C^* - CL) - XQ0_2$ (3)

Where,

X = cell mass concentration

QO_2 = specific oxygen uptake rate

Rearranging the terms we get,

$CL = (-1/ KLa) . \{(dCL/dt) + xQ0_2\}$ (4)

A plot of CL against {(dCL/dt) + xQ02} yields a straight line the negative reciprocal of whose slope gives the value of KLa.

3.2.5.2.. Effect of aeration on KLa

The effect of aeration on the volumetric oxygen transfer coefficient was determined by static gassing out method. The bioreactor was run at different aeration rates of 0.67, 1.34 and 2 vvm and the KLa estimated. The effect of increasing aeration on the KLa was determined by plotting the KLa obtained against the aeration rate.

3.2.5.3. Effect of agitation on KLa

The effect of agitation on the KLa was determined by estimating the KLa at different agitation rates (50, 100, 150 and 200 rpm). The KLa obtained by means of static gassing out method was plotted against the agitation rate.

3.2.5.4. Effect of cell concentration on KLa

To check the effect of the volumetric oxygen transfer rate KLa was estimated by dynamic gassing out method at different time intervals. The cell mass present before the start of the gassing out period and after the gassing out period was checked. Cell concentration was obtained by the average of both these values. Here the agitation was kept at 200 rpm and the aeration at 1.33 vvm. The KLa obtained was plotted against the cell concentration (mg/ml) and the effect determined.

3.2.6 Reverse phase high performance liquid chromatography

The amount of mandelic acid formed in the reaction mixture was also estimated by analytical high performance liquid chromatography (Shimadzu 10AD VP, Kyoto, Japan). Mandelic acid, mandelamide and mandelonitrile were assayed on

LiChroCART® RP-18 column (250 ×4 mm , 5 µm) (Merck, Darmstadt, Germany) at a flow rate of 0.8ml/min with solvent system of water , methanol and phosphoric acid (59.9: 40: 0.1, v/v). The retention time of mandelamide, mandelic acid and mandelonitrile were 4.4, 6.1 and 10.2 respectively. Absorbance was monitered at 210 nm. Two separate linear curves for mandelic acid and mandelonitrile were prepared, from which amount of mandelic acid formed and residual mandelonitrile in the reaction mixture were calculated.

3.2.6.1. Chiral HPLC

The optical purity of mandelic acid was determined by analyzing the enantiomers on Chiracel OD-H column (250 × 0.46mm, 5µm) (Diacel Chemical Industries, New Jersy, USA) at a flow rate of 0.5 ml/min with mobile phase containing hexane, isoporopanol and tri-fluroacetic acid (90:10:0.2, v/v). The retention times for (S)-(+)-isomers and (R)-(-)-isomers were 15.5 and 17.5 min, respectively. Absorbance was monitored at 210nm.

3.2.7 Immobilization methodology

Harvested cells were weighed at a cell mass concentration of 40 mg/ml and dissolved in 10 ml of 100 mM Tris buffer. Different concentration of alginate were taken and dissolved in 10 ml Tris buffer and heated in a microwave oven until alginate fully dissolved in buffer. This hot mixture was cooled to 40^0 C and added to cell slurry with vigorous stirring, after full dispersion of cell into matrices, alginate beads were formed by passing through a syringe and collected in ice chilled calcium chloride solution (0.2 M), beads were put in refrigerator for 30 minutes for hardening (30). After hardening beads were transferred to reaction vessel containing 10 ml Tris buffer, finally substrate (1-acetonapthone) was added in concentration of 30mM and samples were collected at regular intervals for 3hr.

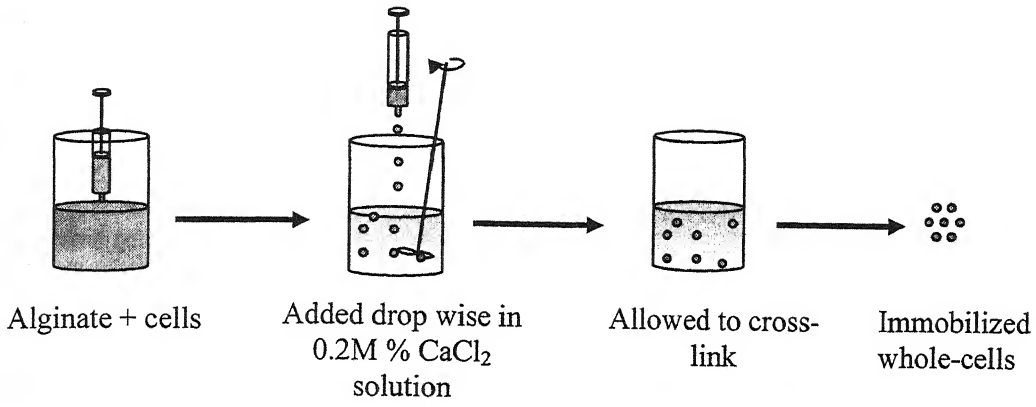


Fig.7. Immobilization methodology for sodium alginate

3.2.7.1. Optimization of immobilization parameters

All the optimization studies were carried out in 50 ml shake flask with 10 ml buffer in doublet with substrate concentration of 30 mM

3.2.7.2 Optimization of matrix concentration

In order to optimize the matrix concentration, different concentration of matrix were used ranging from 1.0, 1.5, 2.0, 2.5 and the cells were immobilized using these matrix concentrations and reaction was carried out at 37°C and 200 rpm. The effect of matrix concentration on immobilized cells was checked by analyzing conversion efficiency and leaching of the cells from matrix. Samples were collected at regular interval for 3 hr and checked for conversion using RP-HPLC.

3.2.7.3. Optimization of bead diameter

For the optimization of bead diameter, various size of beads were prepared using sterile syringe. After hardening the sizes of beads were measured using varnier caliper the sizes of bead formed was 3.456, 4.30, 4.756, 5.256 and 5.746 mm respectively. The reaction was carried out using these beads of different diameter at 37°C and 200 rpm. The samples were collected at regular interval and checked for conversion using RP-HPLC.

3.2.7.4. Optimization of cell concentration

For the optimization of cell loading, different amounts of cell concentration were used i.e. 5, 10, 20, 40, and 60 mg/ml. Cell suspension was prepared with this cell concentrations Immobilization was done to carry out the reaction. Samples were collected at regular interval and checked for conversion using RP-HPLC.

3.2.8 Optimization of reaction parameters for the conversion of mandelonitrile to mandelic acid

The effect of different reaction parameters on the conversion of mandelonitrile to mandelic acid by resting cells of *Escherichia coli* BL21 (DE3) was investigated in order to maximize the productivity of mandelic acid. Cells were harvested after 4 h of growth in the production medium and suspended in phosphate buffer (0.1 M) to obtain resting cell suspension. Biocatalytic reaction was initiated after addition of mandelonitrile to the suspension of resting cells.

3.2.8.1. Effect of pH

To study the effect of pH on mandelonitrile conversion, biocatalytic reactions were carried out in phosphate buffer (0.1 M) of varying pHs ranging from 6.0 to 8.0 and 8.6 (0.1M Tris buffer) at 37°C and the enzyme activity was estimated as described earlier.

3.2.8.2. Effect of temperature

Effect of temperature on *E. coli* catalyzed mandelonitrile conversion was examined by incubating the cell suspension with substrate at various temperatures ranging from 25 to 45°C. The pH of the reaction mixture was kept at optimum value and the enzyme activity was determined.

3.2.8.3. Effect of cell mass loading

Effect of cell concentration on mandelic acid production was investigated by reacting a fixed concentration of mandelonitrile with varying concentration of resting cell suspension (2.5 to 20 mg/ml). The pH and temperature of the reaction mixture were kept at their optimum values as obtained in earlier experiments and liberated ammonia was estimated as previously discussed.

3.2.8.4. Effect of substrate concentration

Optimum substrate concentration for the conversion of mandelonitrile to mandelic acid by *E. coli* cells was determined by reacting different concentration of substrate (10 to 100 µl of 200 mM solution in methanol) with optimum cell concentration. Other reaction conditions were maintained at their optimum values as determined before and the enzyme activity was estimated.

Results & Discussion

4. RESULTS AND DISCUSSION

4.1 Enhancing the production of nitrilase from *Escherichia coli* BL21 (DE3) by medium engineering

Medium components and culture conditions play an important role in the formation of desirable enzymes. Among many methods to improve enzyme activity and yield, optimization of medium components and cultivation conditions remain a facile and feasible way to enhance the enzyme activity and yield. Proper nutrient medium and cultivation conditions not only help in enhancing the desired activity but also lead to abundant growth of the microbial cells which is desirable if these are to be used for further biocatalytic reaction.

4.1.1 Inducer substitution

While optimizing media components, emphasis was given to look for a cheap nutrient from the economic point of view. Therefore, implementation of a cheap but rich nutrient medium which supports abundant growth as well as higher activity of the desired enzyme is the prerequisite for the development of an economically viable process. Keeping this in mind, IPTG was substituted by lactose, results were very encouraging and almost comparable activity was achieved with lactose.

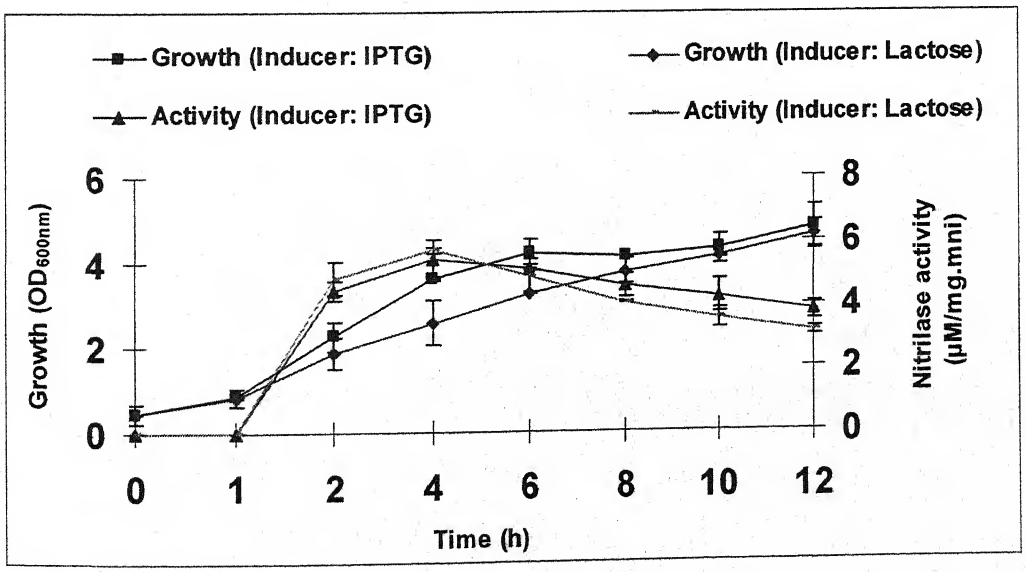


Fig 8. Course of growth and enzyme production with lactose and IPTG as inducer

4.1.2 Substitution of complex media by a Minimal Salt Media (MSM)

After getting a good substitute for the costliest thing we have tried to change media from complex medium to a salt media. Inorganic salts are very cheap as compared to the complex ones. We have tried various factors like MSM with glucose feeding MSM with sucrose feeding, controlled pH strategies etc. As the minimal salt media is not a rich media the activity and growth of *E. coli* was found to be very less as compared to the parent medium and this suggests that some complex media should be tried.

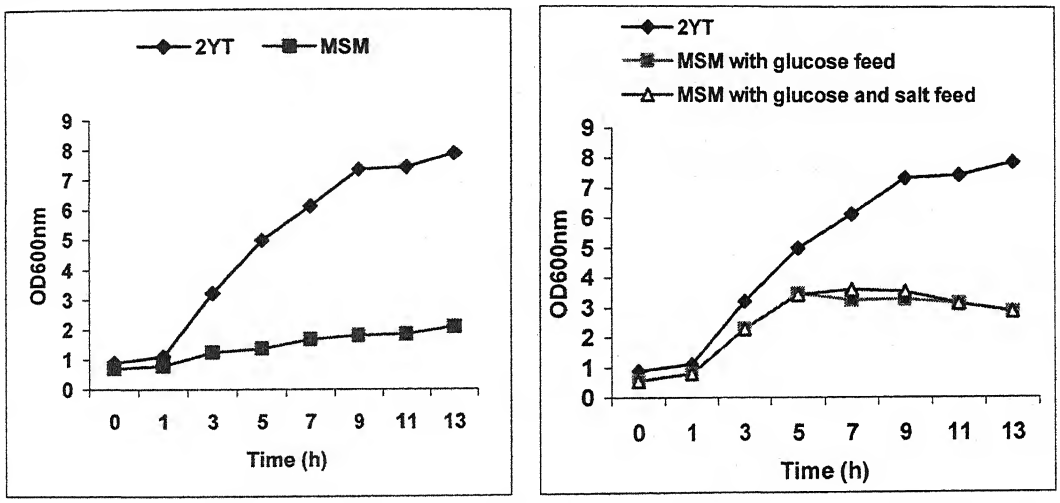


Fig 9 Comparison of growth on complex medium, minimal salt medium, minimal medium with glucose and glucose + salt feeding

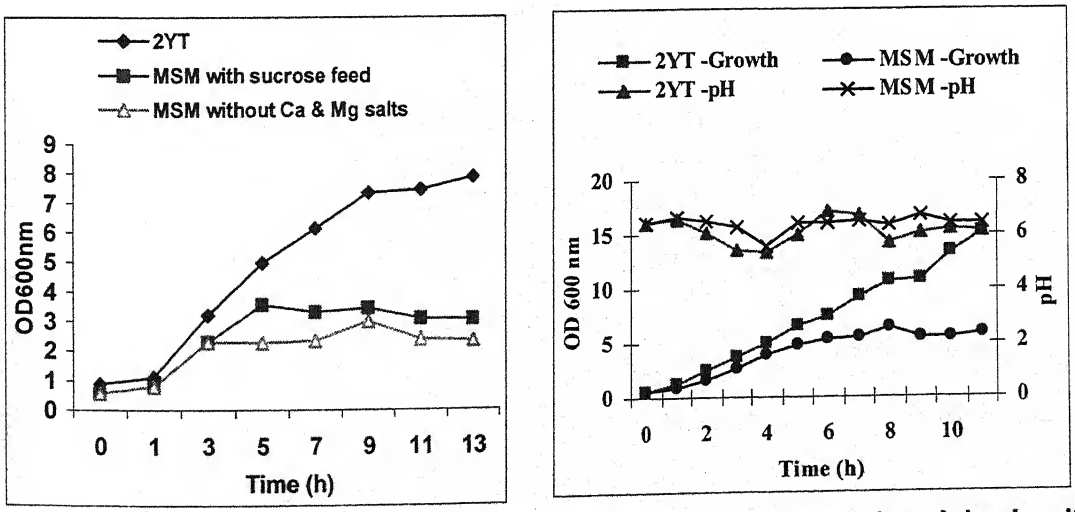
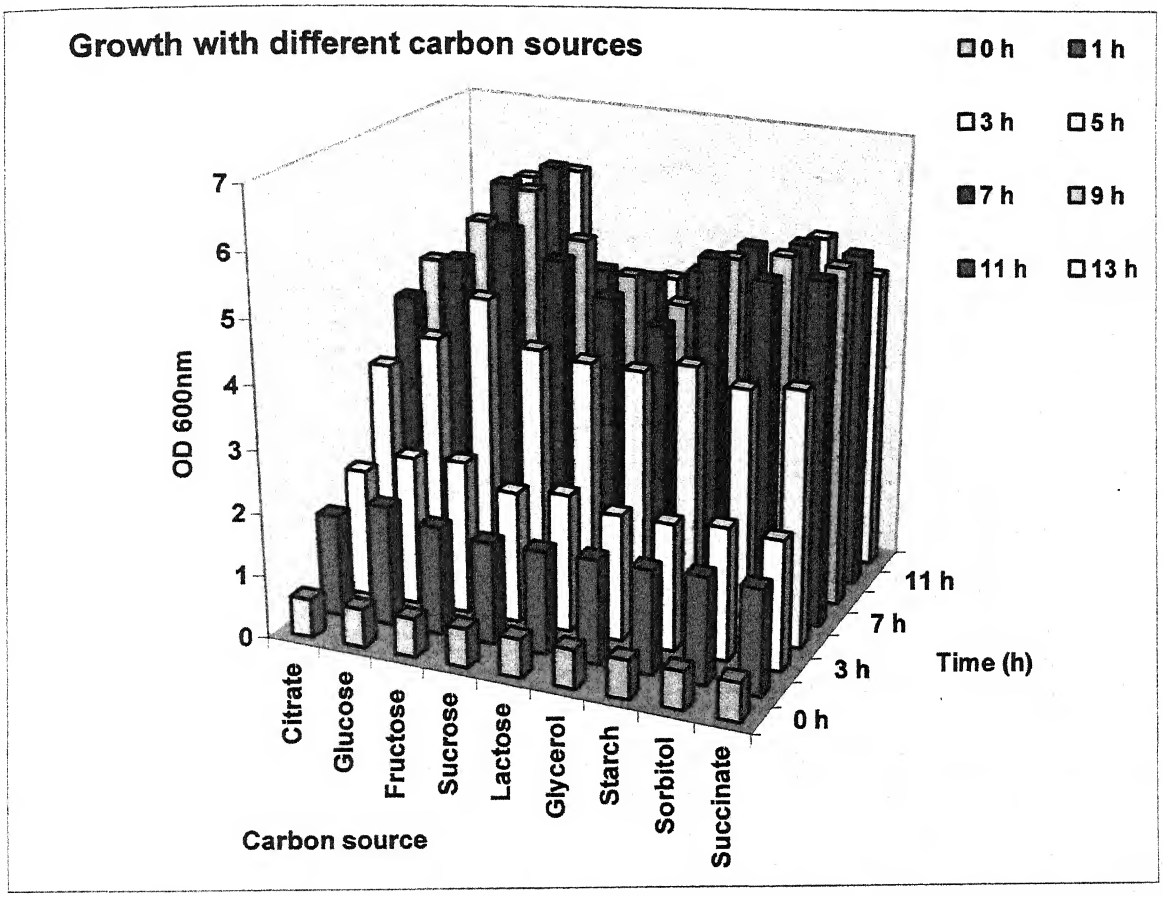


Fig 10 Comparison of growth with sucrose feeding strategies in minimal salt medium, by using MSM without precipitating salts and by precise control on pH



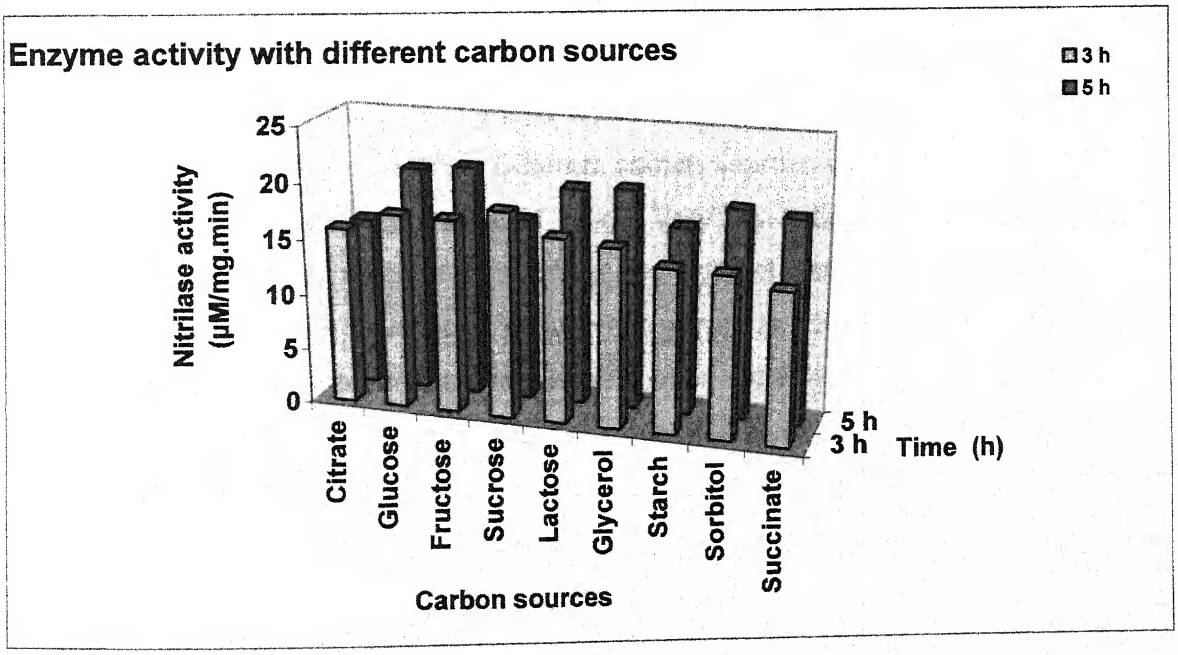


Fig 11 Effect of Carbon source on the growth and enzyme production by *E. coli*.

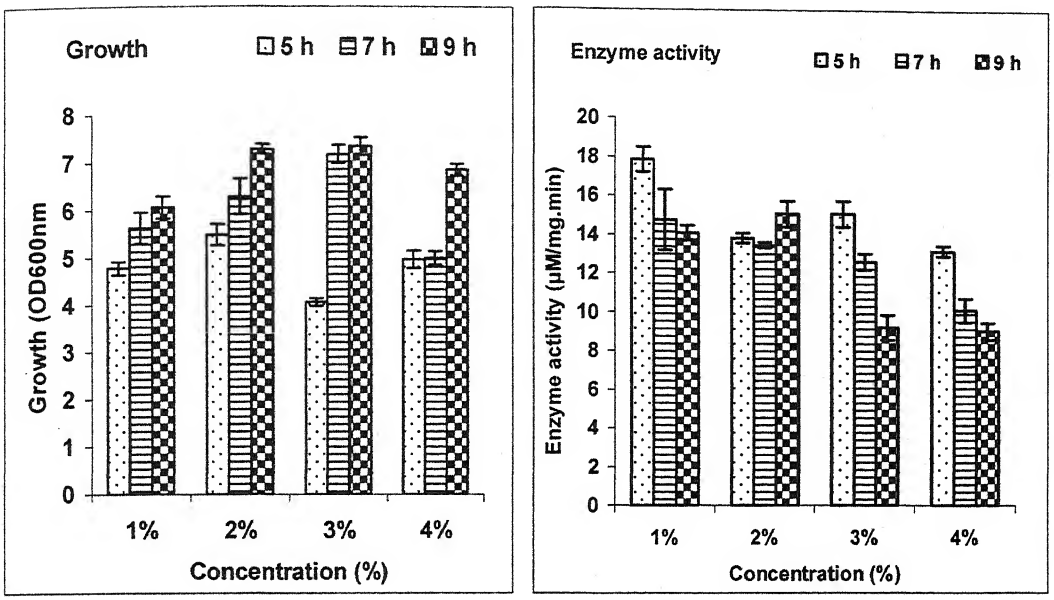


Fig 12. Effect of fructose concentration on the growth and nitrilase production by *E. coli.*

4.1.3 Effect of carbon source

As evident from the experiments with minimal salt medium, we concluded that MSM is not suitable for this particular case and we should optimize the media components of complex medium. Various carbon sources were tested for their effect on growth and enzyme activity. Different carbon sources were added in the basal medium at a final concentration of 10 g/l. Very low activity was obtained when simple sugars such as citrate, succinate and glycerol were used as carbon source. Although sucrose gave good activity but growth is not abundant in this case. Activity as well as growth was comparable when either glucose or fructose is used as carbon source. But due to the fact of catabolite repression associated with glucose we have selected to go with fructose. After this the ideal concentration of fructose for the production of enzyme was establish.

4.1.4 Effect of nitrogen source

Nitrogen source is an important ingredient for the cultivation of microorganisms. Different nitrogen sources, both organic and inorganic, were examined for nitrilase production by *E. coli.* A final concentration of 16 g/l was used. In general organic nitrogen sources being more complex favored higher cell mass

production compared to inorganic nitrogen sources. The inorganic nitrogen sources were not able to produce good enzyme activity compared to organic sources. Among the organic sources tryptone supported higher cell mass production as well as good enzyme activity; hence it was chosen for further studies. Finally concentration of tryptone was optimized.

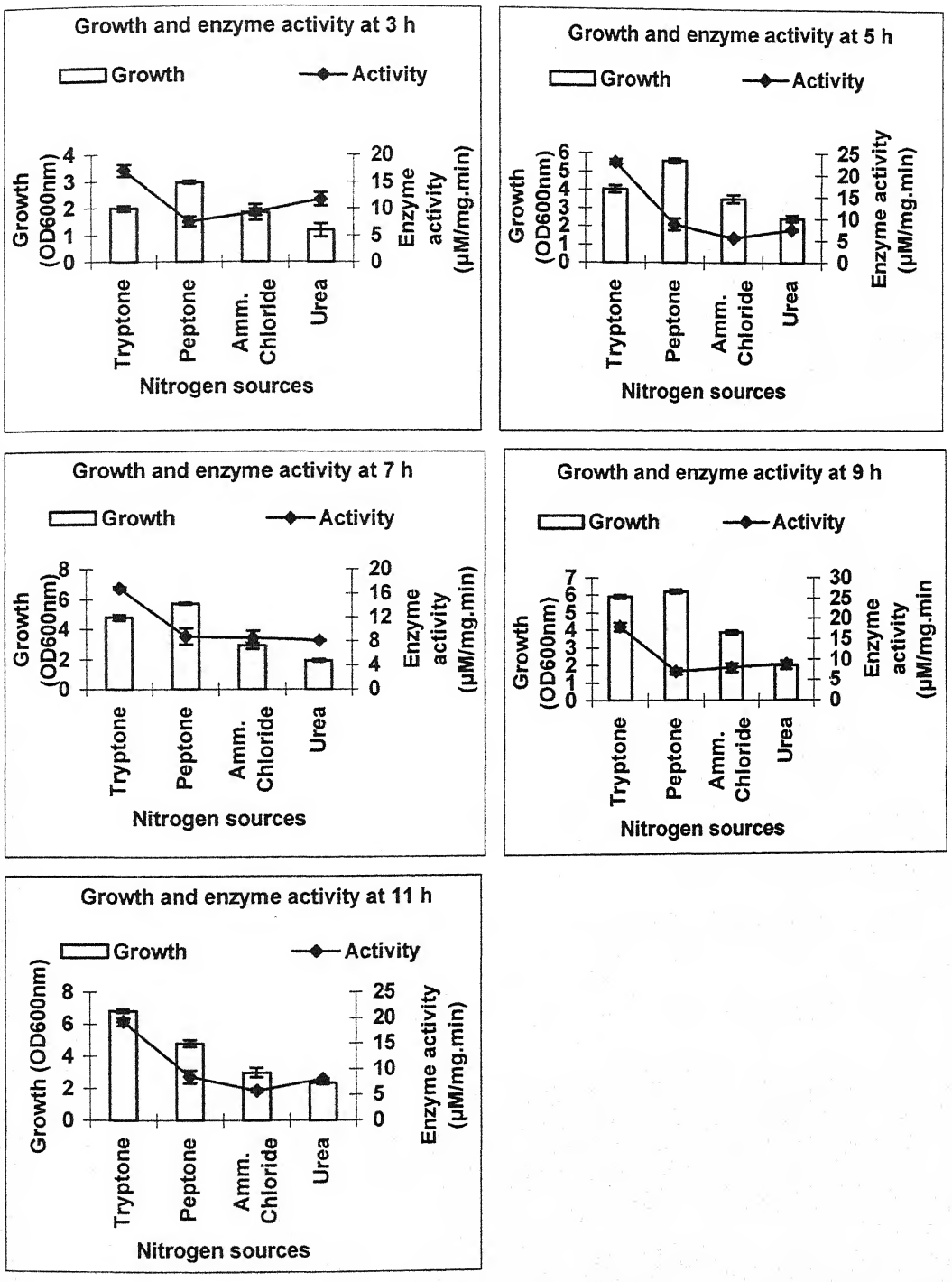


Fig13. Effect of different nitrogen source on the growth and nitrilase production by *E. coli*.

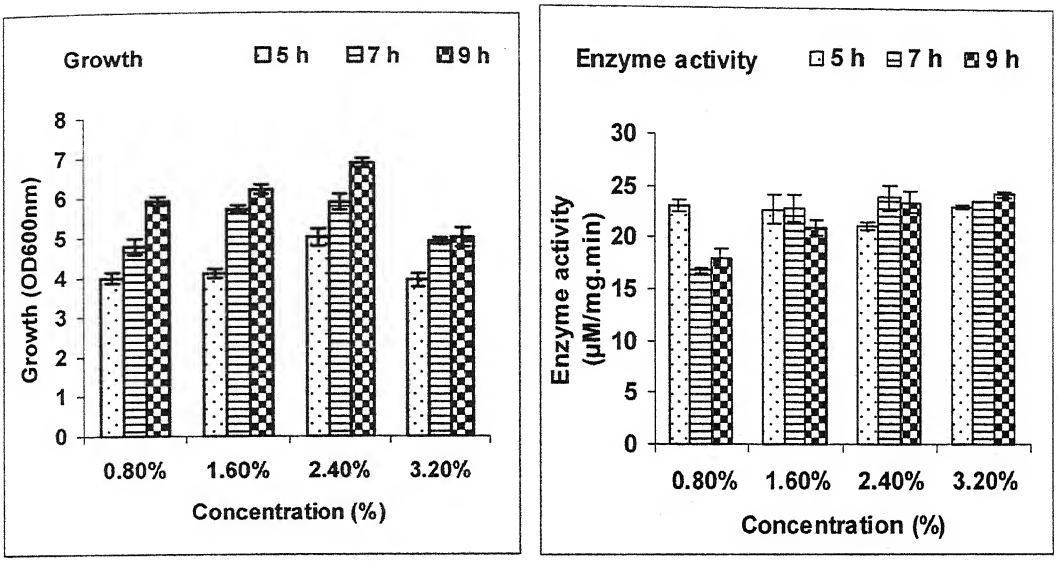
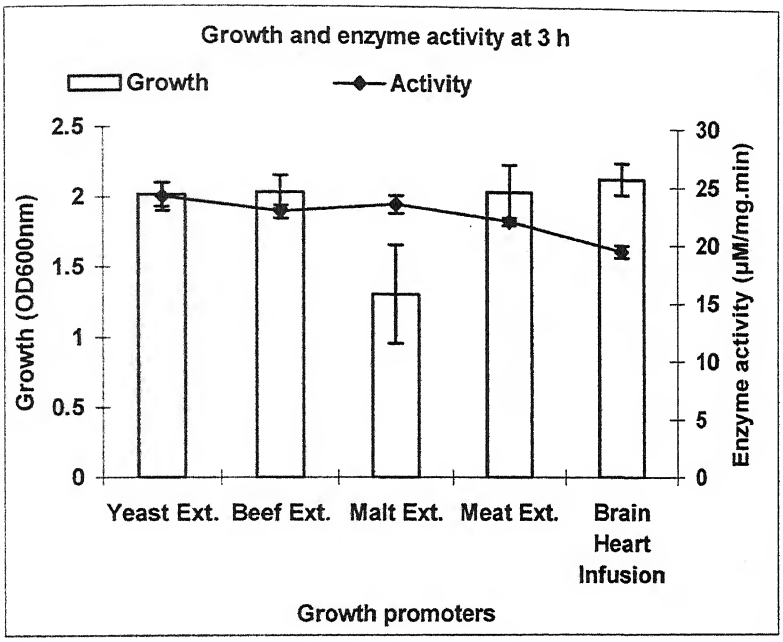


Fig 14. Effect of tryptone concentration on the growth and nitrilase production by *E. coli*.

4.1.5 Effect of growth factor

To enhance the cell mass production, different growth factors like beef extract, yeast extract, brain heart infusion, malt extract and meat extract (final concentration 10 g/l) were examined for their ability to induce maximum nitrilase productivity as well as maximum cell mass growth. It was observed that malt extract, meat extract and yeast extract produced good enzyme but the cell mass production is comparably less in malt extract. As evident from the graph, yeast extract proves to be the best growth promoter, and its concentration was optimized in the next step.



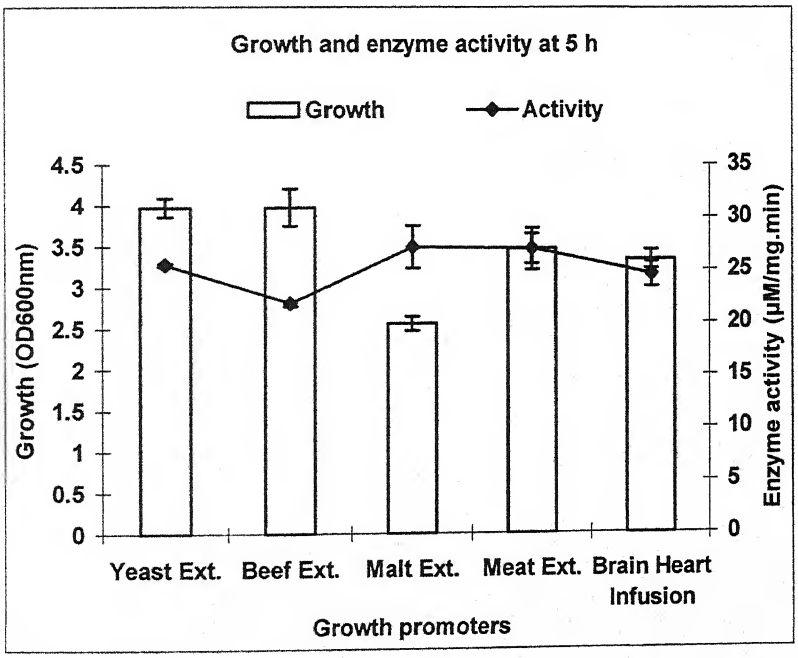


Fig 15. Effect of different growth promoters on the growth and nitrilase production by *E. coli* at 3 and 5h.

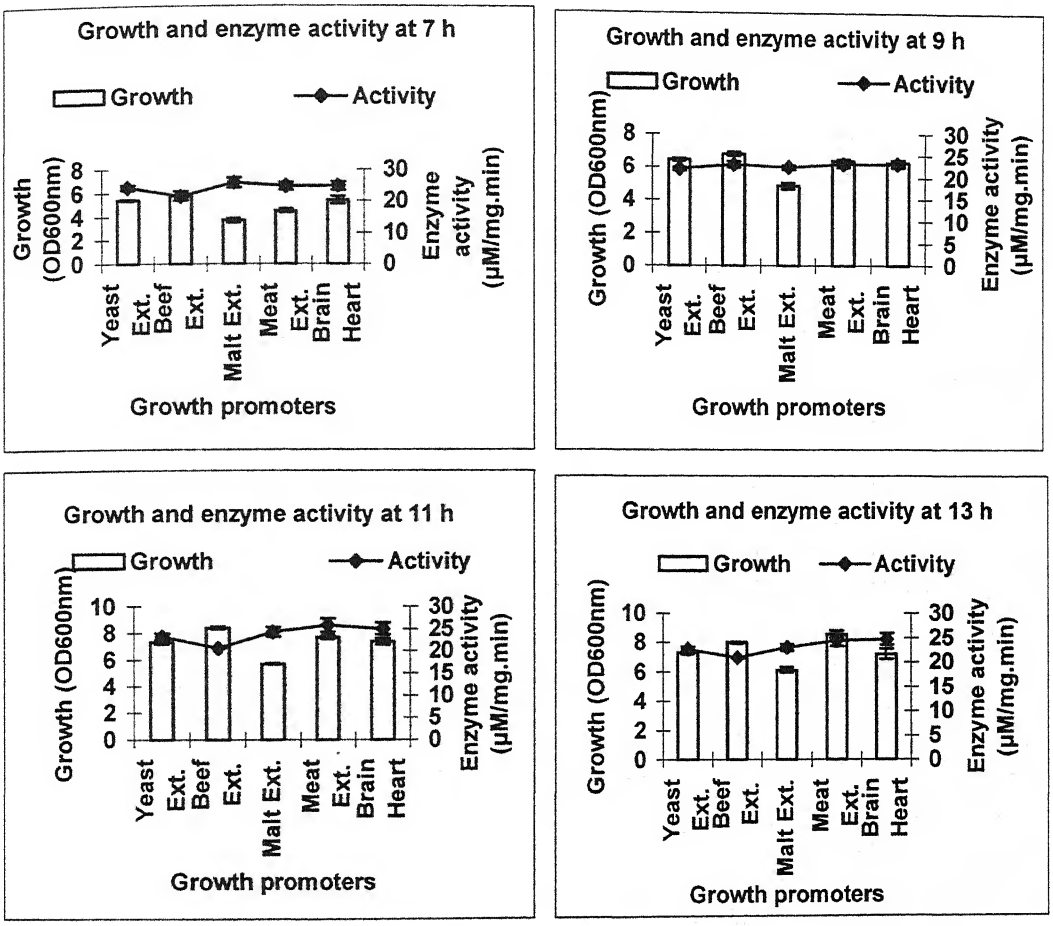


Fig 16. Effect of different growth promoters on the growth and nitrilase production by *E. coli* at 7, 9, 11 and 13 h.

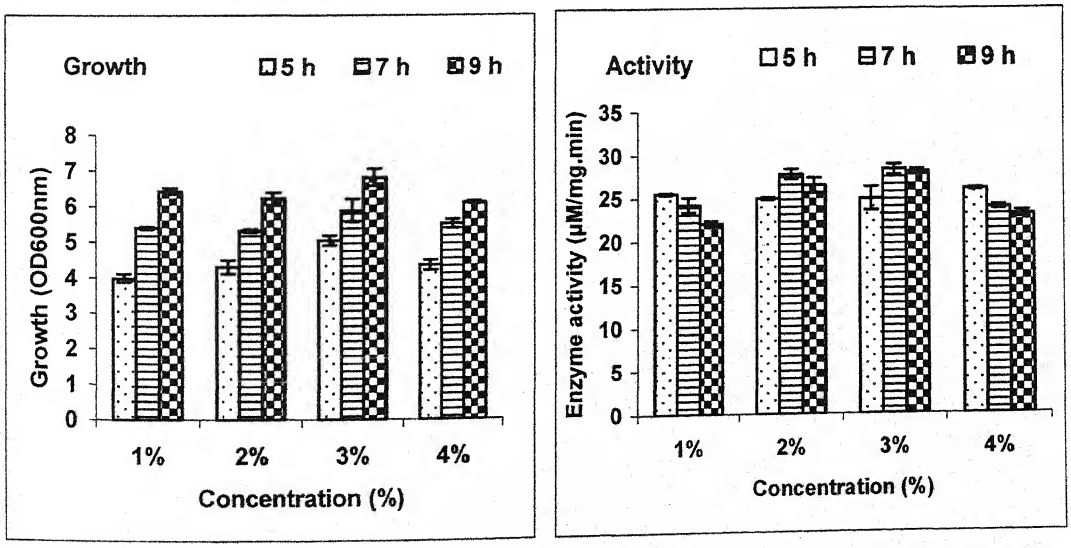


Fig 17. Effect of concentration of yeast extract on the growth and nitrilase production by *E. coli*.

4.2 Effect of environmental factors

The effect of temperature on the growth and nitrilase production by *E. coli* was studied in the basal media as described earlier. Cells were allowed to grow at different temperature and samples were taken at regular interval and analyzed. It was observed that growth is almost comparable at all the temperature except 40°C however; nitrilase production is maximum at 37°C.

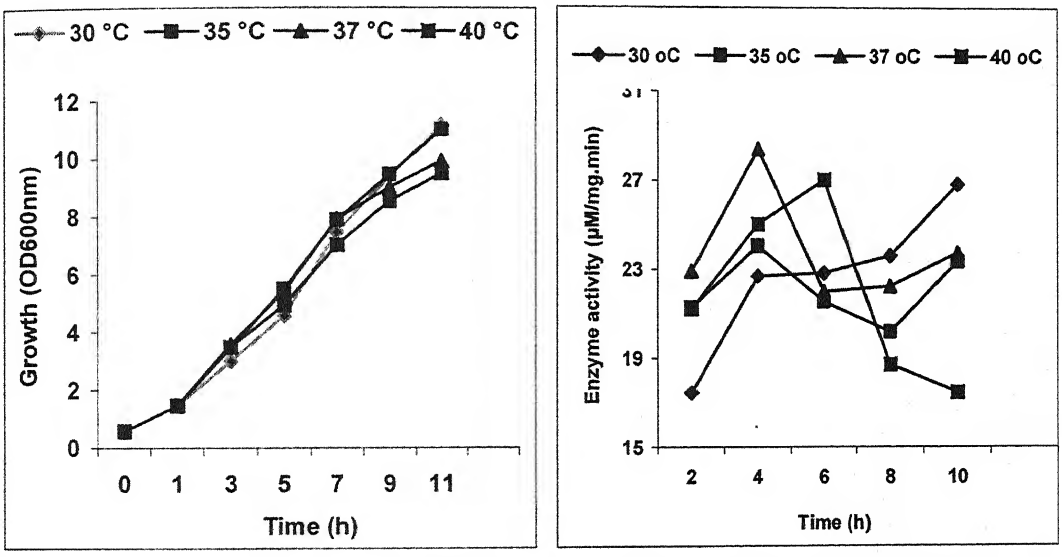


Fig. 18 Growth and nitrilase production by *E. coli* at different temperatures

4.3. FERMENTER STUDIES

4.3.1. Effect of initial pH

To examine the effect of initial pH on the growth and nitrilase production by *E. coli,* cultivation was carried out at different initial pH (6.5 - 8.5) in the bioreactor. At an initial pH 7.5, it was observed that the organism multiplied and produced nitrilase at the highest level. Deviation on the either of the side of the optima leads to fall in enzyme activity and growth.

Initial pH = 6.5

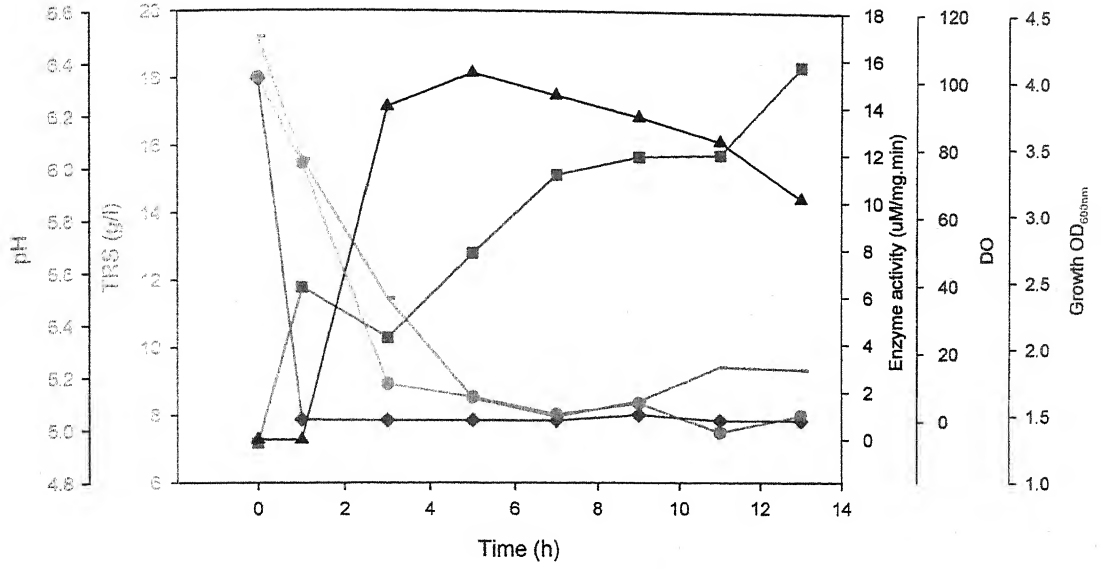


Fig 19. Course of growth and nitrilase production at an initial pH of 6.5 by the recombinant *E. coli* in 2 liter bioreactor. [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

Initial pH = 7.5

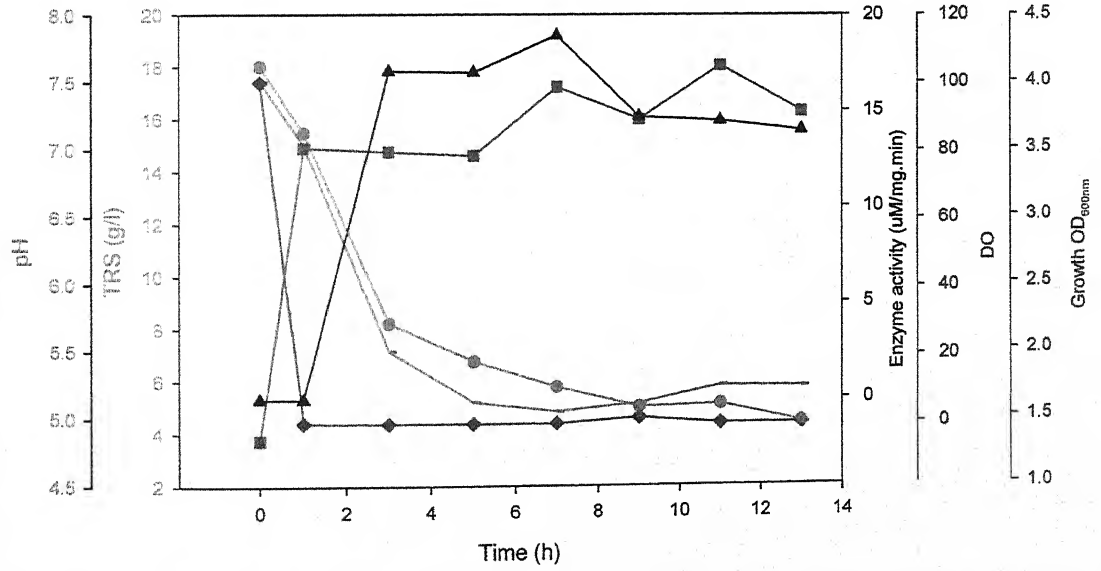


Fig 20. Course of growth and nitrilase production at an initial pH of 7.5 by the recombinant *E. coli* in 2 liter bioreactor. [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

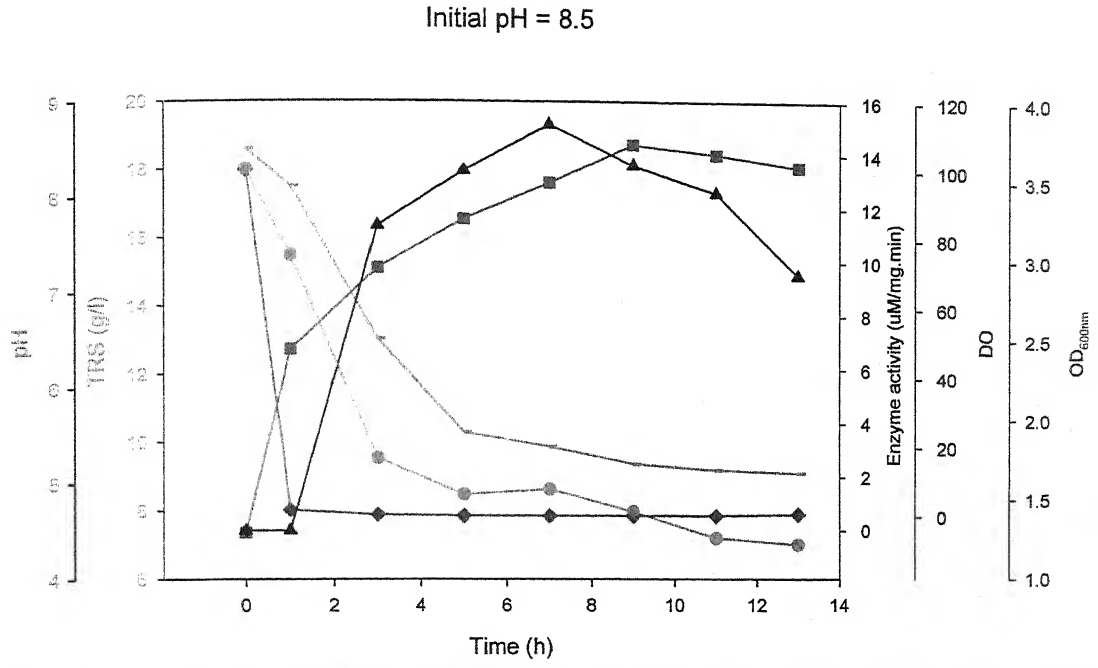
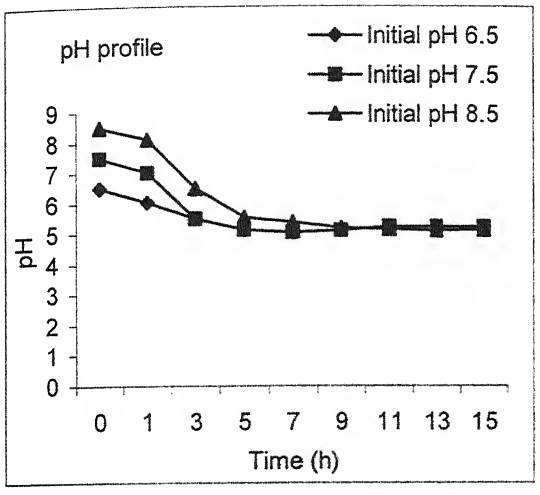
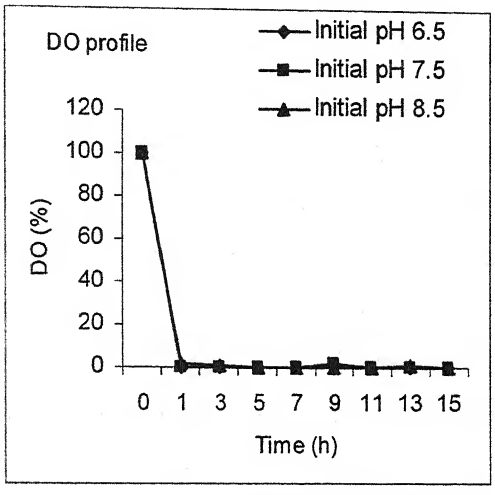
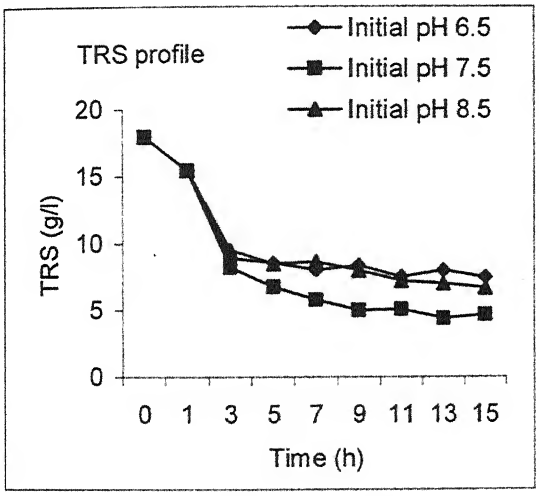
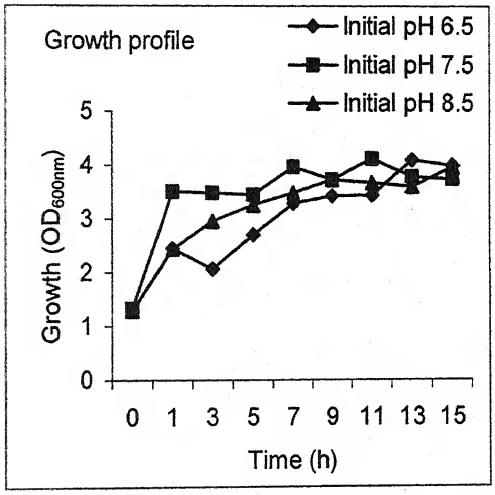


Fig 21. Course of growth and nitrilase production at an initial pH of 8.5 by the recombinant *E. coli* in 2 liter bioreactor. [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].









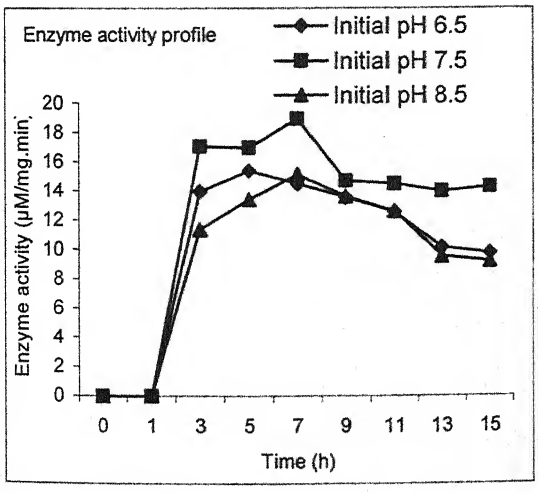


Fig 22. Variation of pH, DO, total reducing sugar (TRS), growth & enzyme activity at different initial pH.

4.3.2. Effect of agitation

Agitation is important for proper mixing, mass transfer and heat transfer. Agitation of the culture broth can have a variety of effects on microorganisms. These effects may be deleterious or beneficial and include rupture of the cell wall, change in cell morphology, variation in the efficiency and rate of growth, and variation in the rate of formation of the desired product. Cell rupture generally occurs when local shear forces (near the impeller region) in the vessel are higher than the forces necessary to break cell wall. Also there is the problem of excess foam being generated at higher mixing speeds. Investigations were carried out at different agitational speeds of 200, 300 and 400 rpm for the production of nitrilase by *E. coli.* It was observed that with the increase of agitation, growth of *E. coli* has increased but at the same time enzyme activity decreased. Most probable reason for such a phenomenon may be that the sulphydryl group of the active site may have been oxidized due to increased oxygen in the bioreactor.

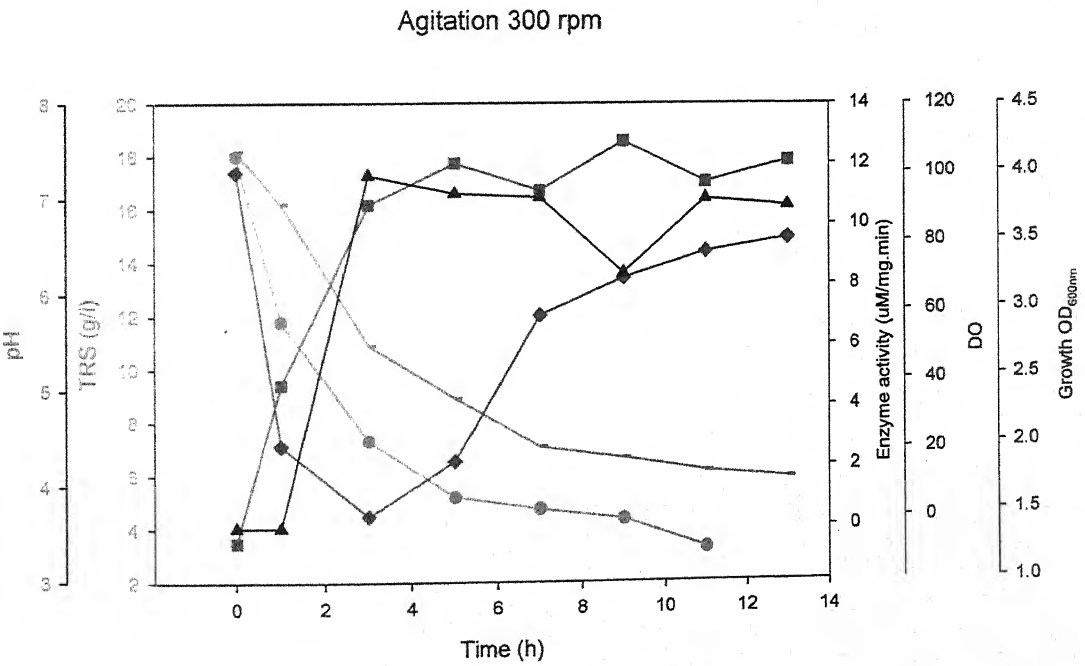


Fig 23. Course of growth and nitrilase production at a mixing rate 300 rpm by the recombinant *E. coli* in 2 liter bioreactor. [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

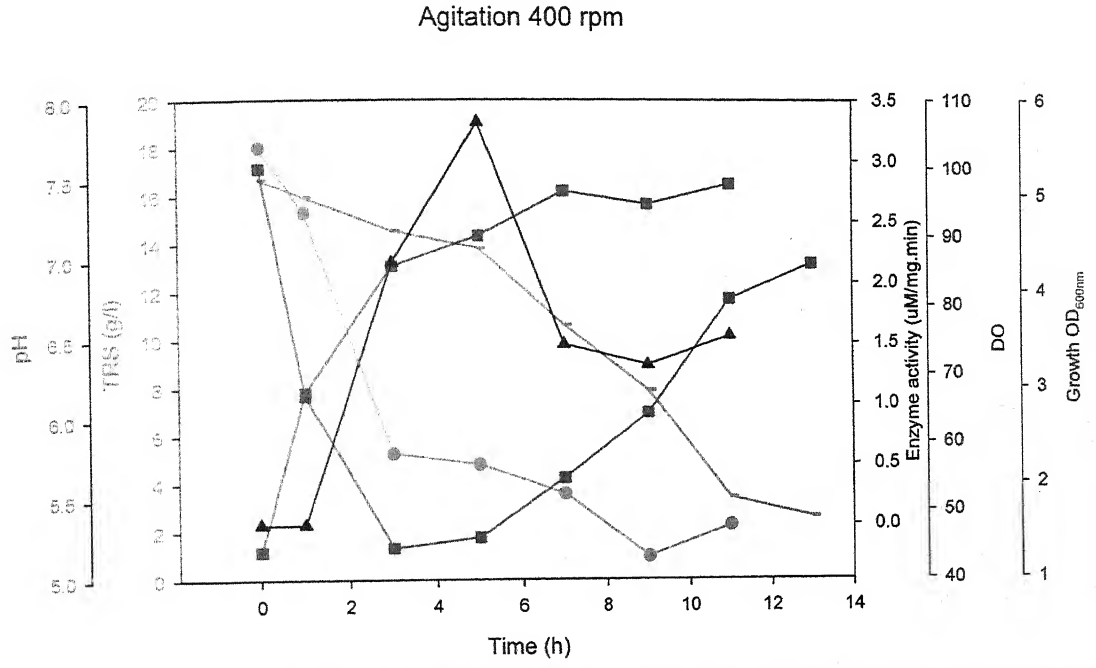


Fig 24. Course of growth and nitrilase production at a mixing rate 400 rpm by the recombinant *E. coli* in 2 liter bioreactor. [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

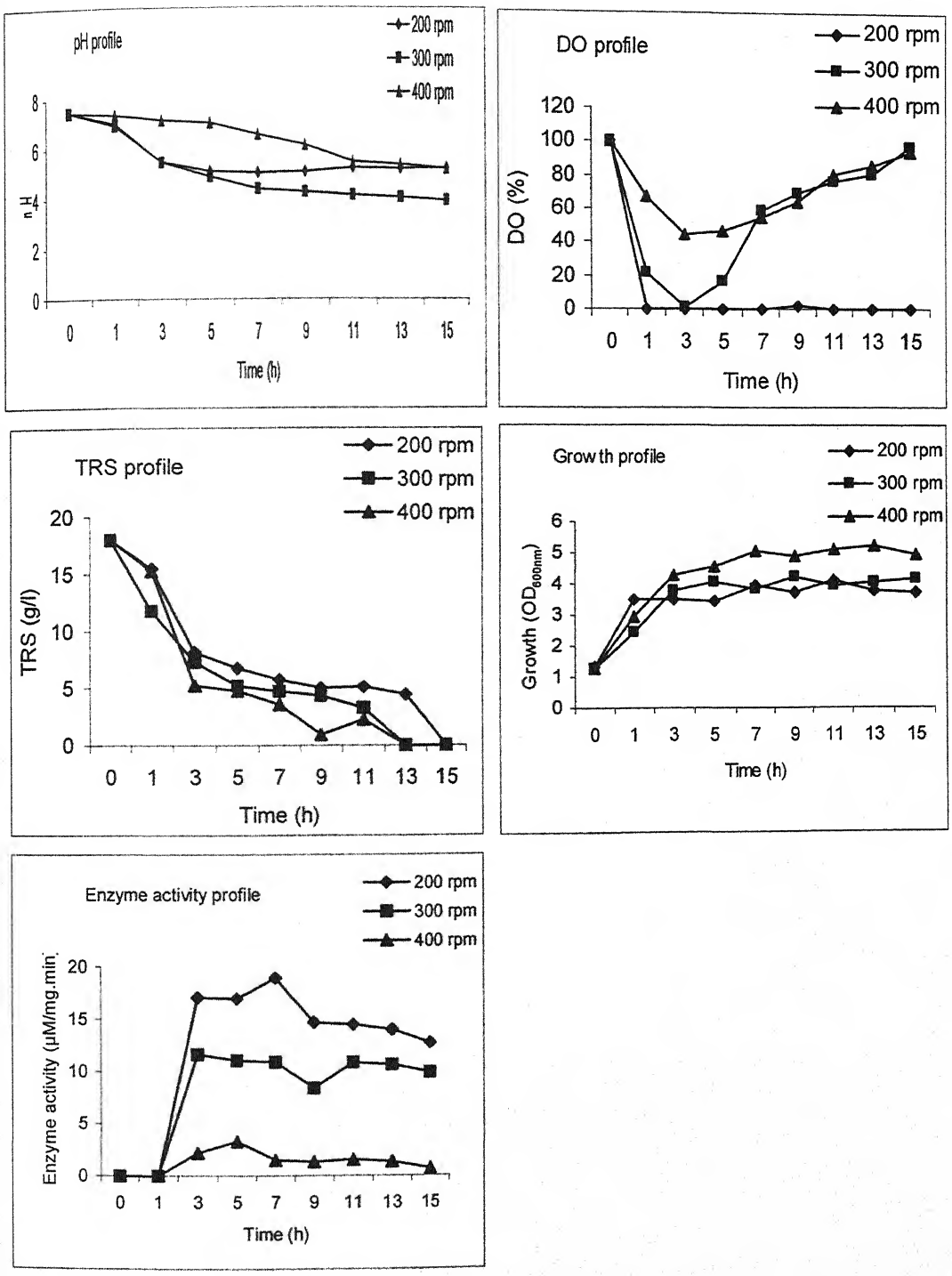


Fig 25. Variation of pH, DO, total reducing sugar (TRS), growth & enzyme activity at different agitational condition.

4.3.3. Effect of aeration

Aeration is essential for metabolic activities of aerobic organisms. At the same time, aeration also brings about mixing of the bioreactor contents thereby increases the efficiency of the bioreaction. The concentration of dissolved oxygen in a suspension of respiring microorganism generally depends on the rate of oxygen transfer from the gas phase to the liquid phase, rate at which oxygen is transported to the site of utilization and its consumption by the microorganism. During fermentation, the transfer of oxygen occurs from an air bubble into the liquid phase, gets transferred to the cell and ultimately to the site of oxygen reaction in a cell particle. This study of oxygen transfer from air bubbles, through the liquid medium, to microbial cells is of great importance. Oxygen transfer can be increased by increasing the aeration rate. The effect of increasing the aeration rate was checked by sparging the bioreactor with air at different aeration rates (0.5, 1 and 1.5 vvm). Almost same phenomenon was observed as with the agitation that the growth increases with aeration but activity decreases.

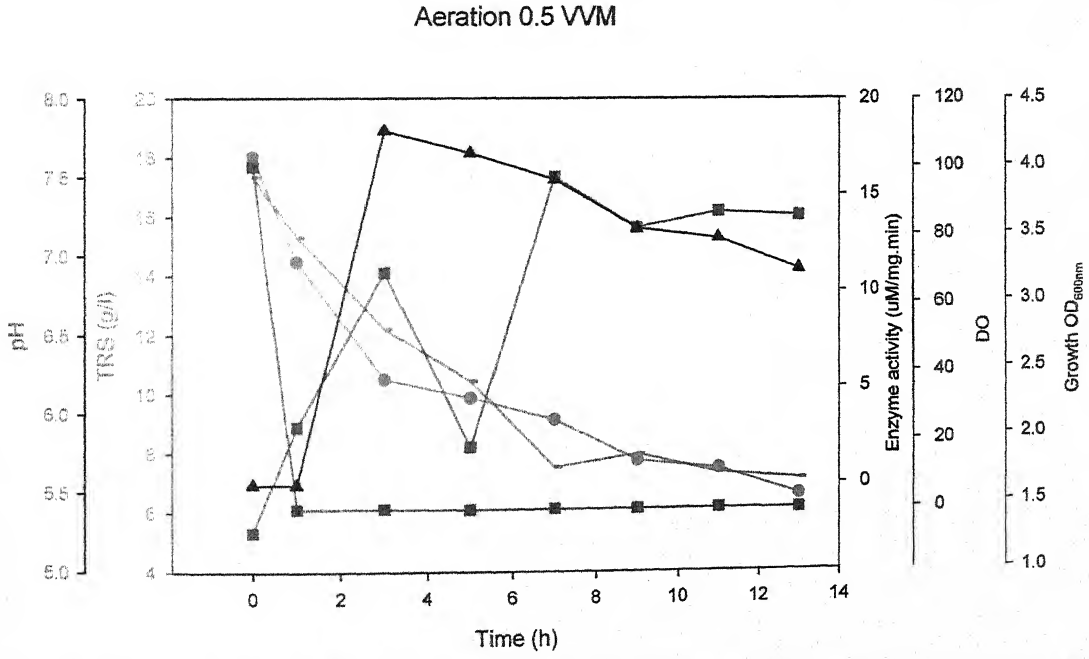


Fig 26. Course of growth and nitrilase production at a 0.5 vvm aeration for the recombinant *E. coli* in 2 liter bioreactor [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

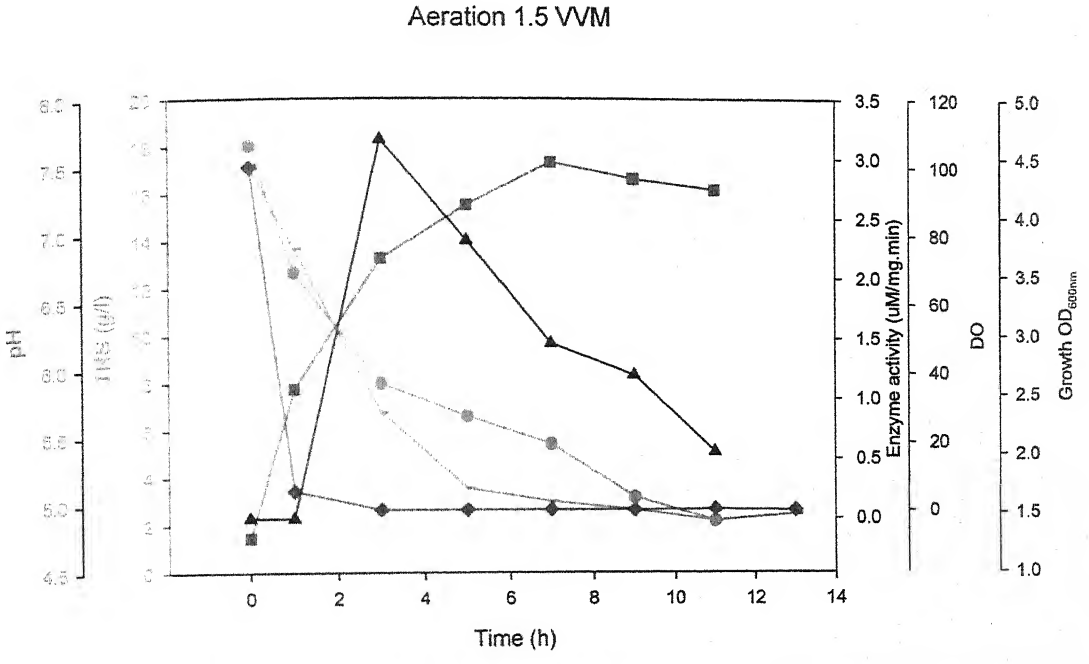
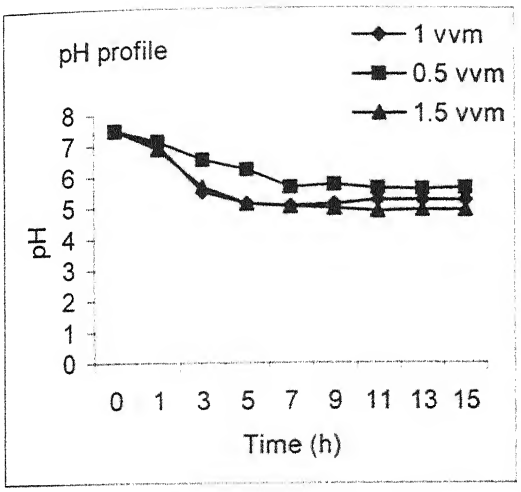
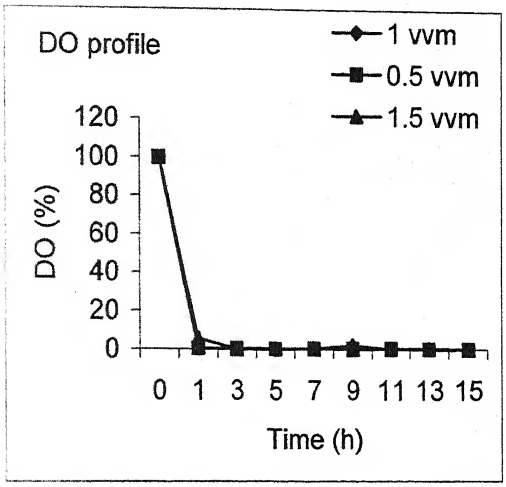
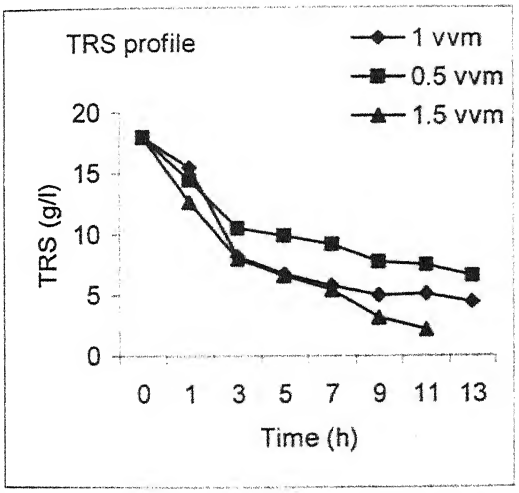
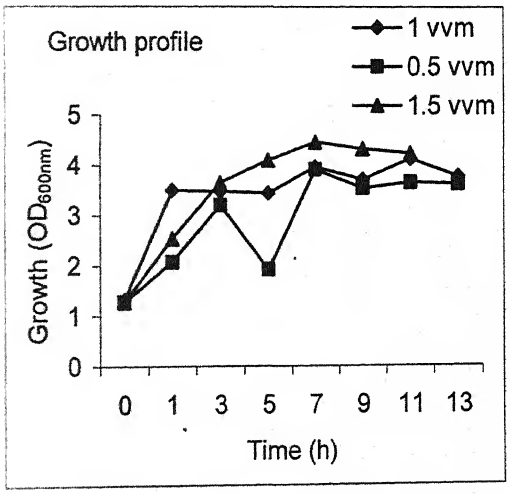


Fig 27. Course of growth and nitrilase production at a 1.5 vvm aeration for the recombinant *E. coli* in 2 liter bioreactor [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].









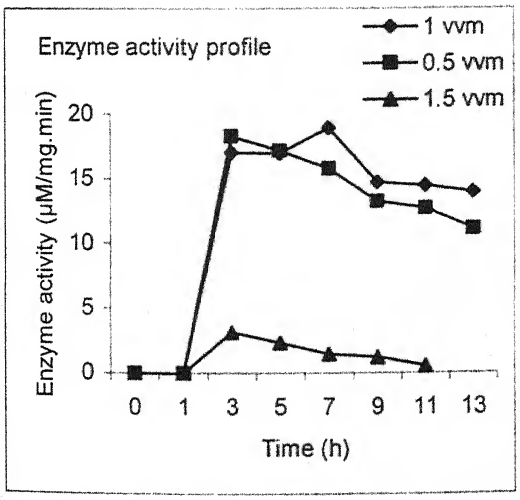


Fig 28. Variation of pH, DO, total reducing sugar (TRS), growth & enzyme activity at different aeration condition.

4.3.4. Effect of controlled pH

Since the pH of the medium was found to decrease throughout the cultivation regardless of the initial pH of the medium, studies were carried out with controlled pH, such as 6.5, 7.5 and 8.5. The pH of the medium was maintained by the addition of 1N H_2SO_4 or 1N NaOH as and when required. The pH was chosen based on the effects observed in the earlier runs. A controlled pH 7.5 was chosen as the maximum specific activity was obtained when the initial pH was maintained at 7.5.

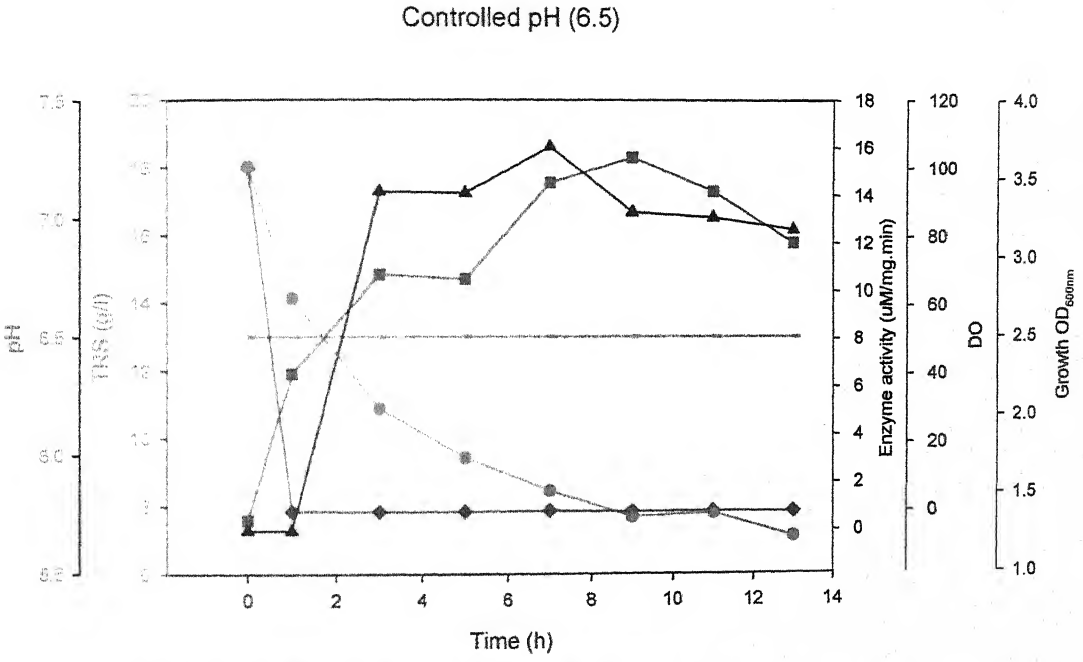


Fig 29. Course of growth and nitrilase production at a controlled pH of 6.5 by the recombinant *E. coli* in 2 liter bioreactor [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

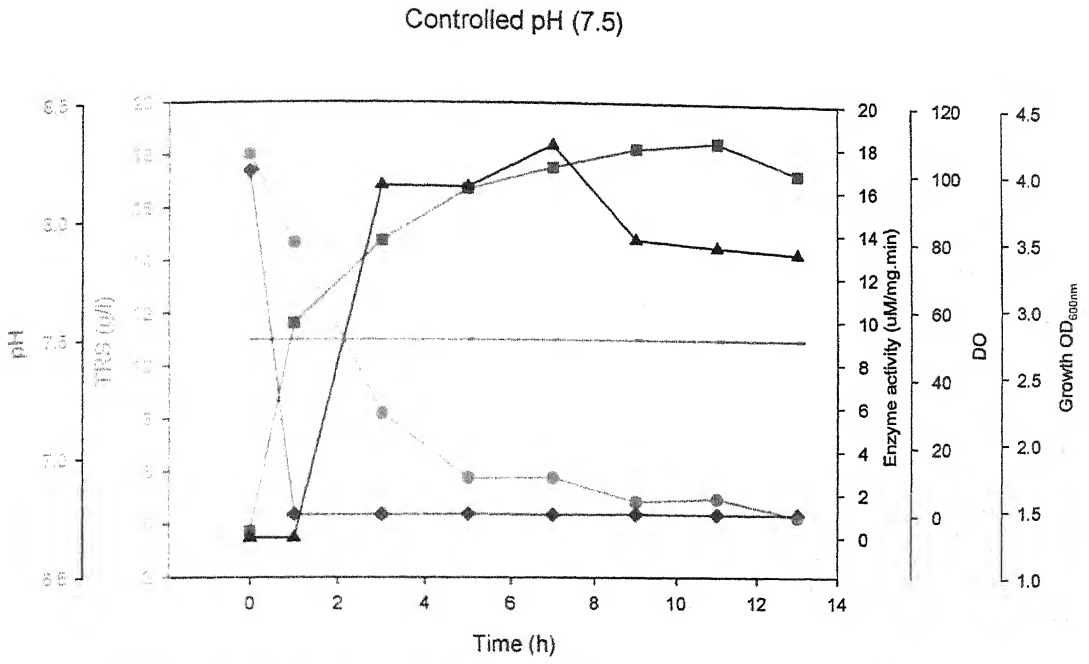


Fig 30. Course of growth and nitrilase production at a controlled pH of 7.5 by the recombinant *E. coli* in 2 liter bioreactor [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

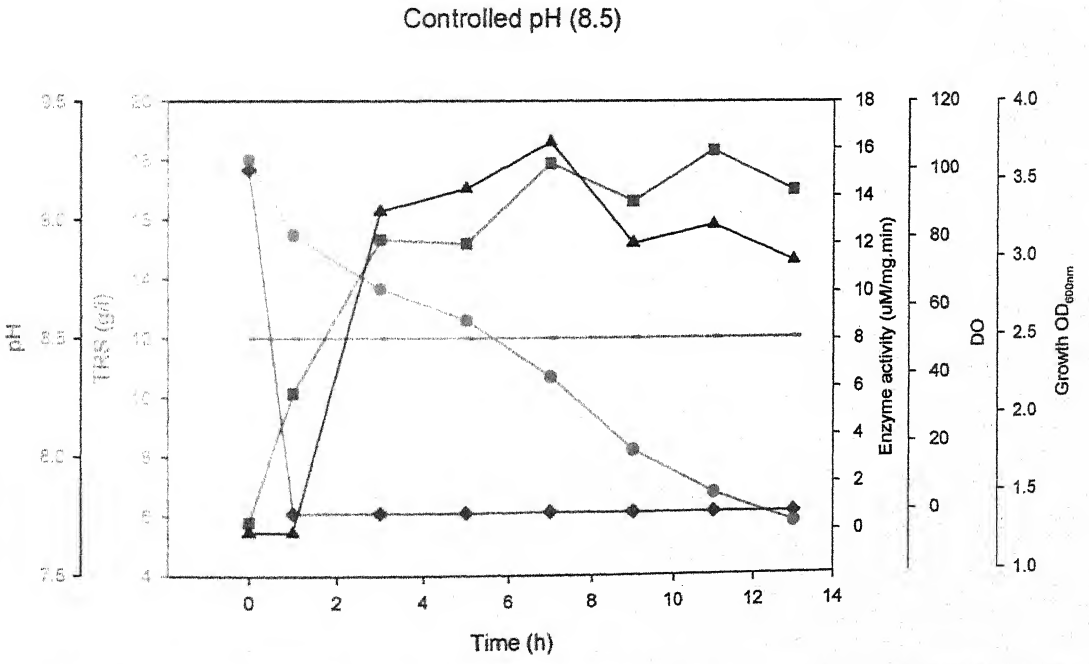


Fig. 31. Course of growth and nitrilase production at a controlled pH of 8.5 by the recombinant *E. coli* in 2 liter bioreactor [TRS (•), pH (–), Activity (▲), DO (◊), OD600nm (■)].

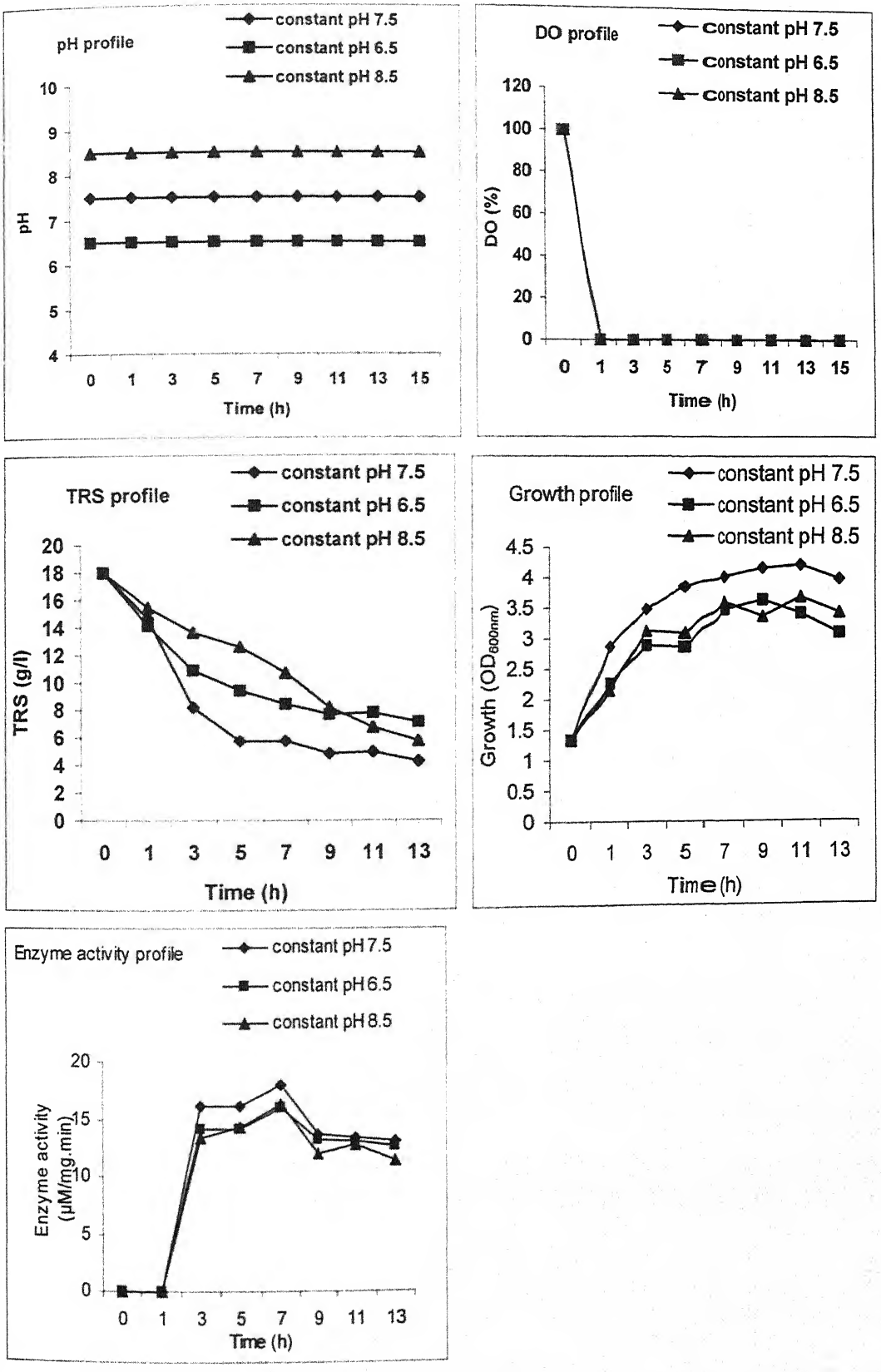
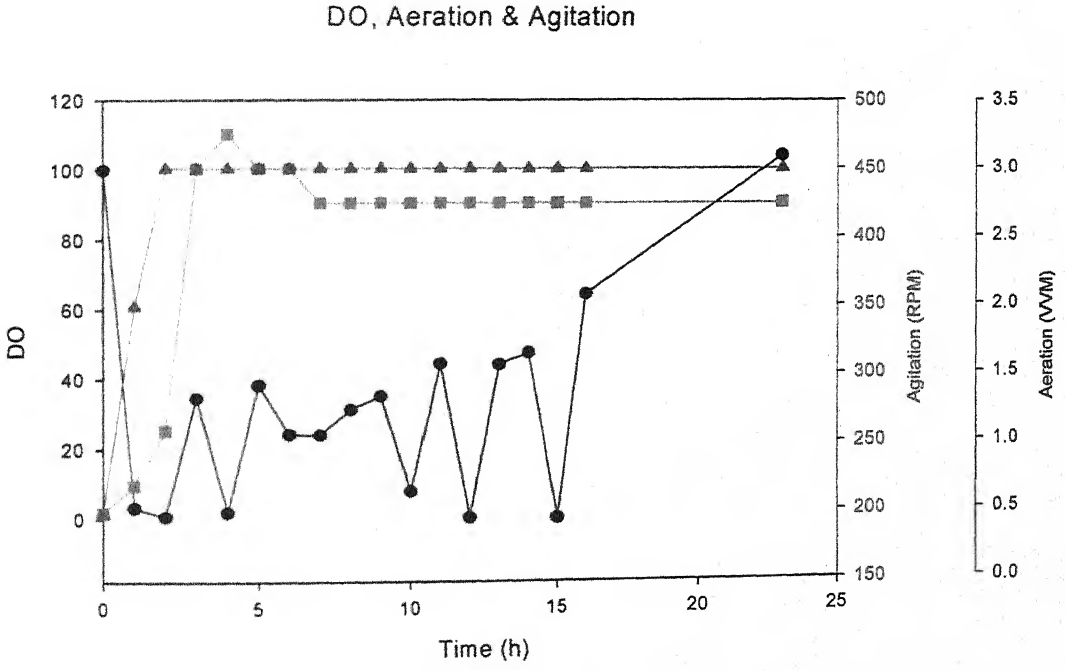


Fig 32. Variation of pH, DO, total reducing sugar (TRS), growth & enzyme activity at different constant pH.

4.3.5. Effect of feeding and increasing DO level

Main purpose of growing cells at higher level in bioreactor is that, it is a closed system and environment can be controlled very precisely. As observed from the above experiments that the organism is demanding more dissolved oxygen for growth and at the same time almost whole fructose is also depleted in the process. Hence it was decided to carry out fructose feeding with increasing dissolved concentration by varying agitation and aeration.

Soon after the first feed run it was observed that early induction is not very beneficial as all the desired enzyme activity is not achieved. Also sugar addition requires a fixed profile. Hence it was decided that induction would be done only after a certain growth level is achieved.



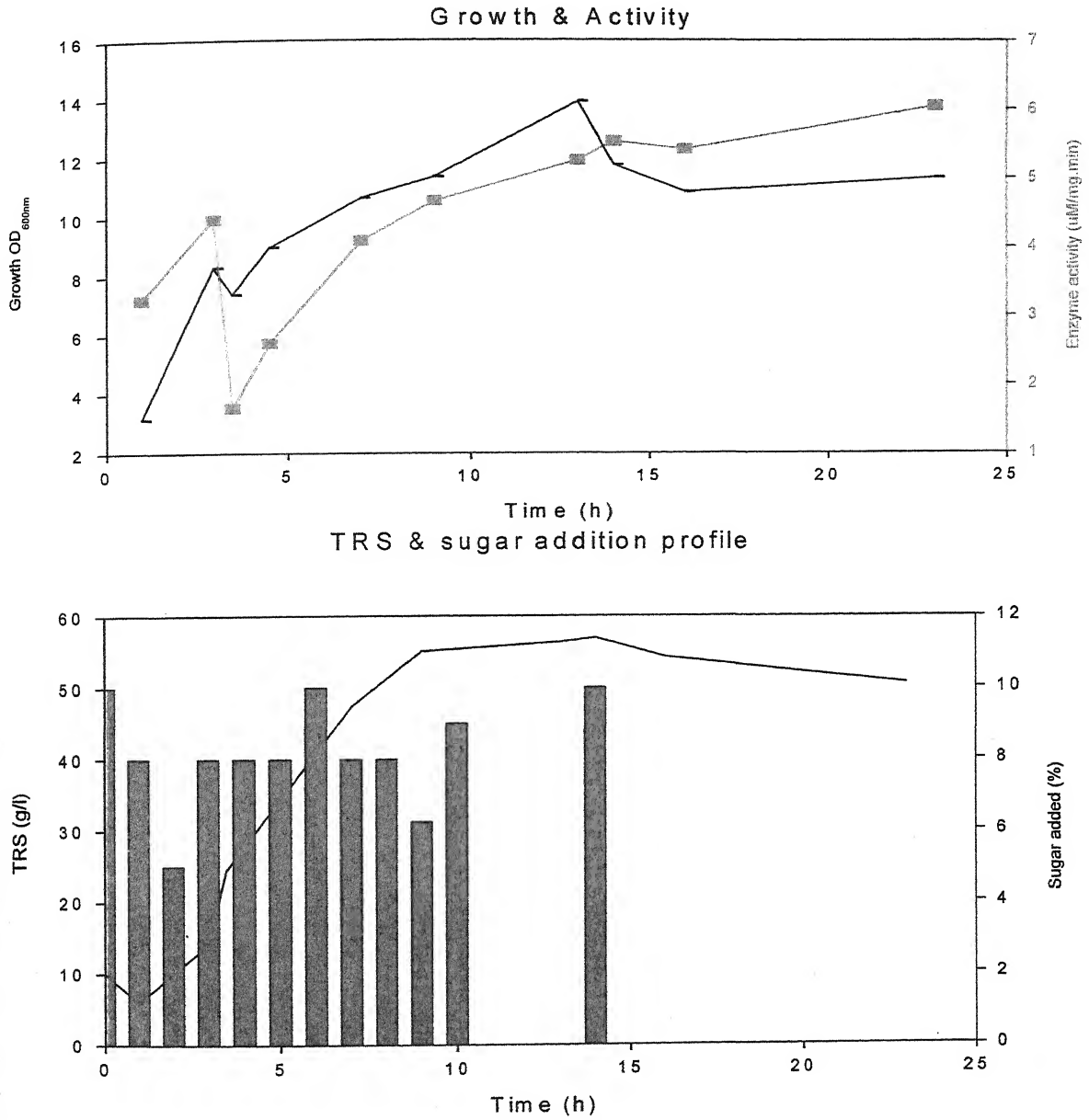


Fig 33. (a) Course of change of DO, agitation and aeration during fermentation of *E. coli* when induction is carried out at 1 h [DO (●), Aeration (▲) and Agitation (■)].

(b) Course of change of growth and enzyme activity during fermentation of *E. coli* when induction is carried out at 1 h [Activity (◊) and Growth (–)].

(c) Course of change of total reducing sugar (TRS) and addition of sugar during fermentation of *E. coli* when induction is carried out at 1 h [line represent TRS & bar represent Sugar addition].

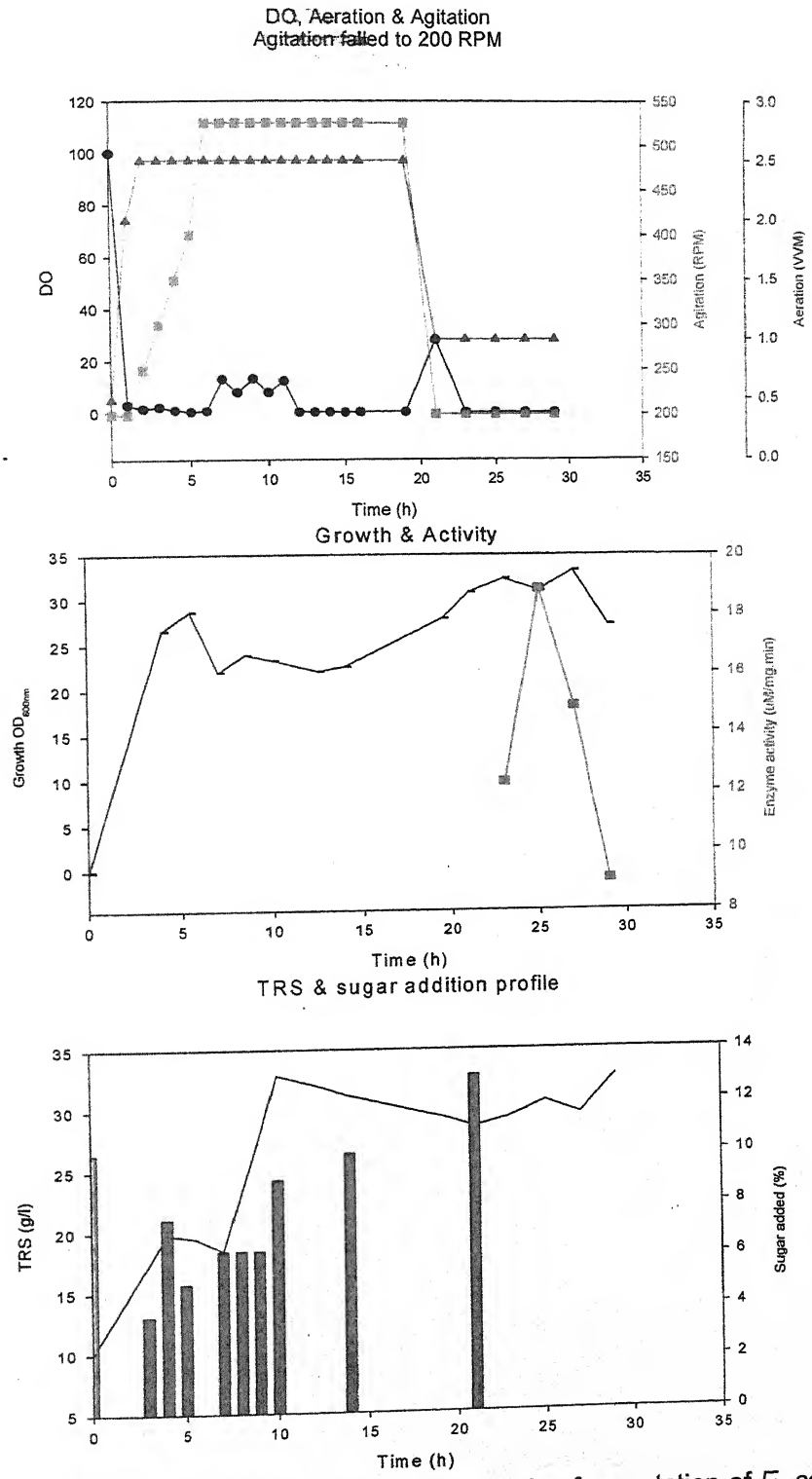


Fig 34. (a) Course of change of DO, agitation and aeration during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 200 rpm and 1 vvm respectively [DO (●), Aeration (▲) and Agitation (■)].

(b) Course of change of growth and enzyme activity during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 200 rpm and 1 vvm respectively [Activity (◊) and Growth (–)].

(c) Course of change of total reducing sugar (TRS) and addition of sugar during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 200 rpm and 1 vvm respectively h [line represent TRS & bar represent Sugar addition].

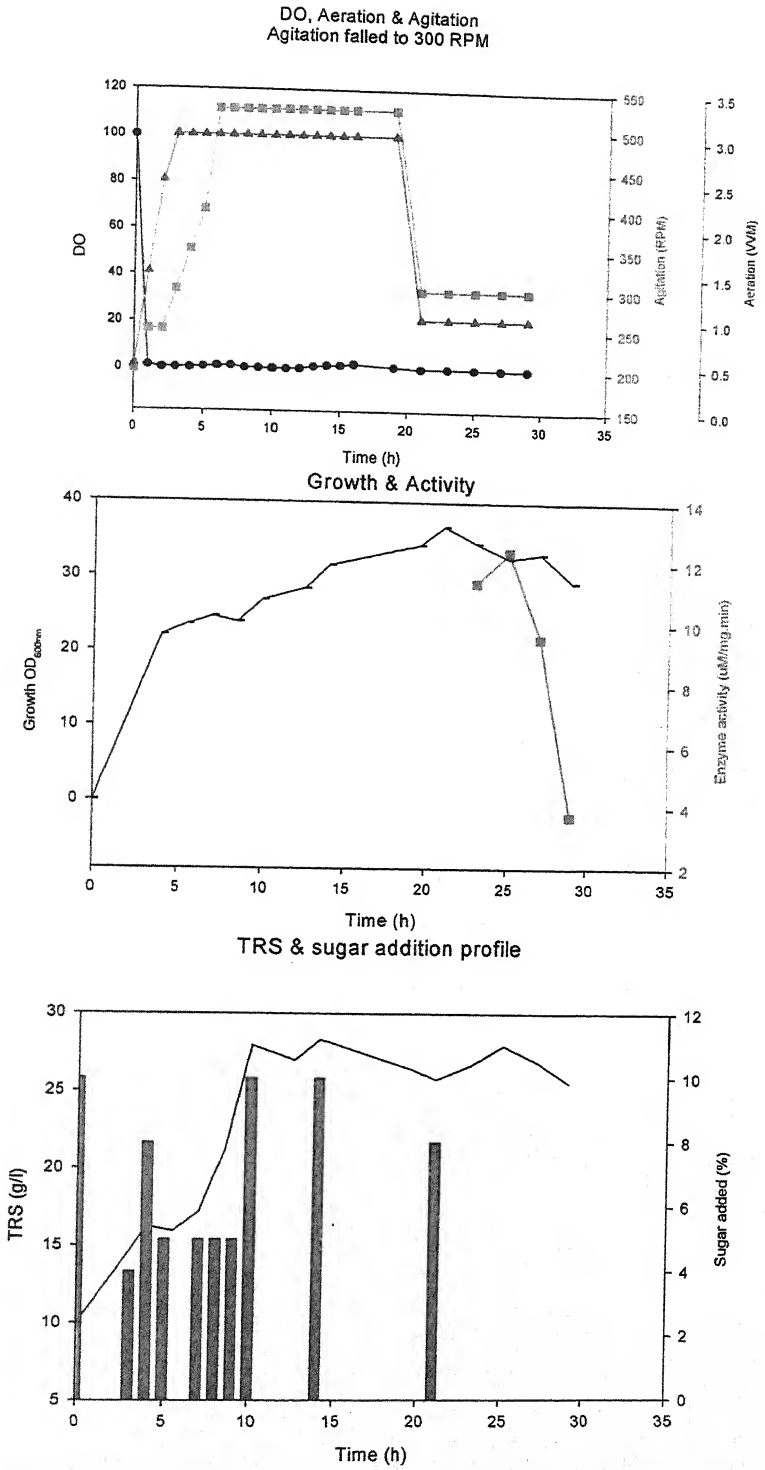


Fig 35. (a) Course of change of DO, agitation and aeration during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 300 rpm and 1 vvm respectively [DO (●), Aeration (▲) and Agitation (■)].

(b) Course of change of growth and enzyme activity during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 300 rpm and 1 vvm respectively [Activity (◊) and Growth (—)].

(c) Course of change of total reducing sugar (TRS) and addition of sugar during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 300 rpm and 1 vvm respectively h [line represent TRS & bar represent Sugar addition].

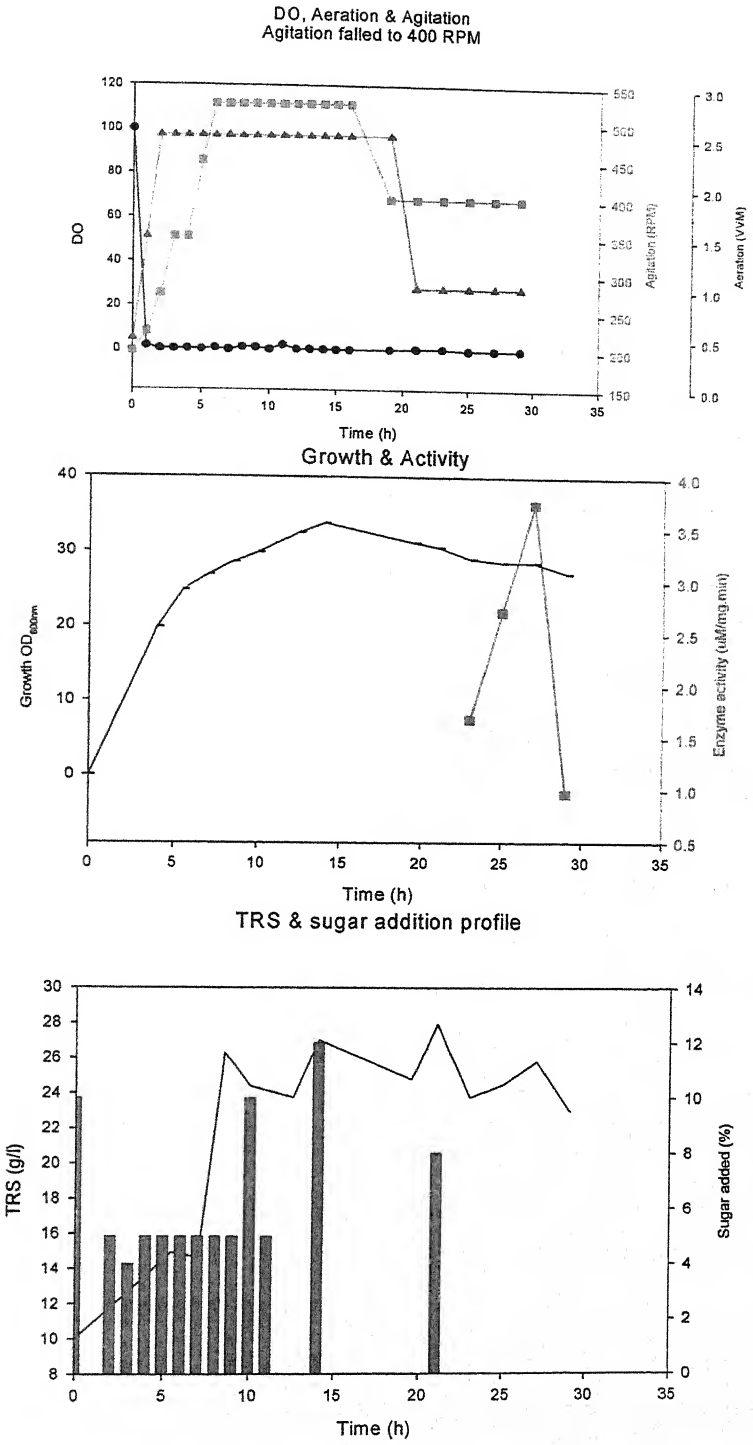


Fig 36. (a) Course of change of DO, agitation and aeration during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 400 rpm and 1 vvm respectively [DO (●), Aeration (▲) and Agitation (■)].

(b) Course of change of growth and enzyme activity during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 400 rpm and 1 vvm respectively [Activity (◊) and Growth (–)].

(c) Course of change of total reducing sugar (TRS) and addition of sugar during fermentation of *E. coli* when induction is carried out at 21 h. Agitation and aeration are adjusted to 400 rpm and 1 vvm respectively h [line represent TRS & bar represent Sugar addition].

4.4.1 Determination of the volumetric oxygen transfer coefficient (KLa) and effects of different parameters on the (KLa)

The transfer of oxygen to the cell occurs via the following steps:

- Transfer of oxygen from air bubble to the liquid.
- Transfer of the dissolved oxygen from the liquid to the cell
- Uptake of the oxygen by the cells

The limiting step is the rate of transfer of oxygen from the air bubble to the liquid. This oxygen transfer rate may be described by equation (1) (Sec 3.2.5).

As it is extremely difficult to measure the actual values of both KL and 'a', hence the two values are combined and is known as the volumetric mass transfer coefficient (KLa), which is a measure of the aeration efficiency of the fermenter. Larger the value more will be the aeration capacity of the system. The value of KLa is unaffected by the oxygen transfer rate but is a function of various parameters like aeration rate, agitation rate and impeller and fermenter design. These variables affect the value of KLa by changing the resistance to mass transfer as well as the number, size and residence time of the air bubbles. As aeration efficiency frequently depends on the KLa values, efforts are made to increase the values to increase the efficiency. This can be done by increasing the value of KL or that of 'a'. The value of KLa depends on the characteristics of the liquid which cannot be manipulated to a great extent. However the values of the interfacial area 'a' can be played around with. This is done by increasing the surface area of the air bubbles which in turn can be achieved either by increasing the aeration or the agitation. The most common methods to increase 'a' in stirred tanks are to increase the agitation rate and to increase the gas sparging rate. Increasing the impeller rate increases impeller shear, thus increasing bubble break-up and increasing the value of 'a'. Smaller bubbles also have a smaller rise velocity, and consequently a greater residence time in the liquid. The longer the bubbles remain in the reactor, more the oxygen transferred. Increasing the sparging rate increases the number of bubbles present in the liquid. To increase the oxygen mass transfer coefficient, the effect of different variables like aeration, agitation rate and cell concentration on KLa was determined by using the gassing

out methods i.e. static and dynamic gassing out methods. Dynamic gassing out is carried out during the logarithmic phase of the growth of the microorganism. However, in the case of recombinant *E. coli* the DO concentration rapidly decreases to zero value. This is because the oxygen utilisation rate of recombinant *E. coli* is very high which leads to complete utilization of the dissolved oxygen in the medium. Hence static gassing out method was used to determine the effects of agitation rate and aeration rate on KLa. The effect of cell concentration was estimated by means of dynamic method. But for this it was necessary to use pure oxygen instead of air for aeration so as tp satisfy the oxygen requirements of the organism.

4.4.1.1 Effect of agitation rate on the volumetric oxygen transfer coefficient

KLa was found to increase linearly with increasing agitation in a range of 50-200 rpm (Fig 37).

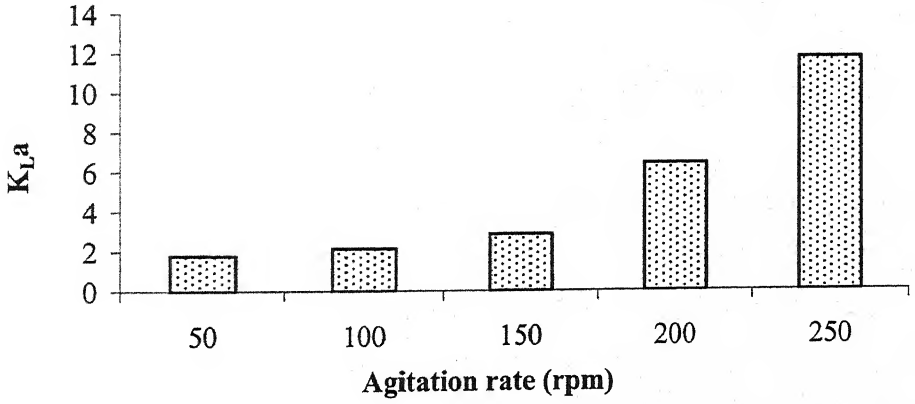


Fig 37. Effect of agitation rate on volumetric mass transfer coefficient (KLa)

The value of the volumetric oxygen transfer coefficient was found to be lowest at 50 rpm and maximum at 200 rpm among the different agitation rates.

4.4.1.2 Effect of aeration rate on the volumetric oxygen transfer coefficient

The volumetric oxygen transfer coefficient was found to increase with the increase in the aeration rate in a range of 0.67 vvm to 2 vvm. Among the given aeration rates the value of KLa was estimated to be the highest at 2 vvm (Fig 38).

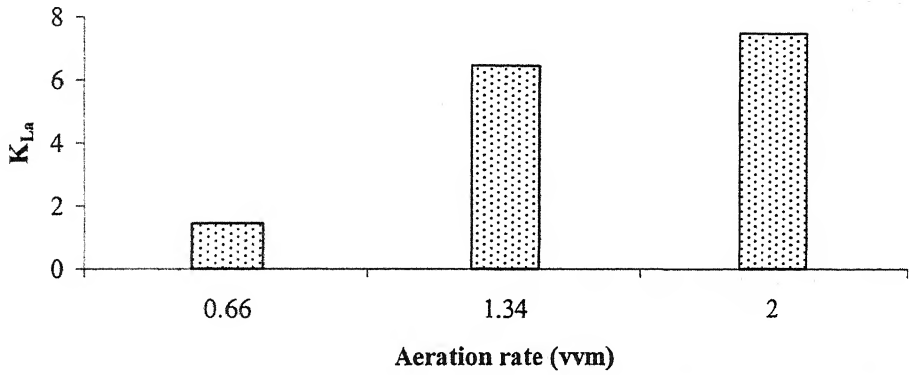


Fig 38. Effect of aeration rate on volumetric mass transfer coefficient (KLa)

4.4.1.3 Effect of cell concentration on the volumetric oxygen transfer coefficient

The effect of cell concentration on KLa was determined by dynamic gassing out method. It was observed that during the 'gassing out' phase the slope of the curve increased with the cell concentration. This is because the slope of the curve represents the specific oxygen uptake rate (XQO2) (Eq. 4) of the organism where (X) is the cell mass concentration. Hence, an increase in the value of X with time, leads to a corresponding increase in the slope

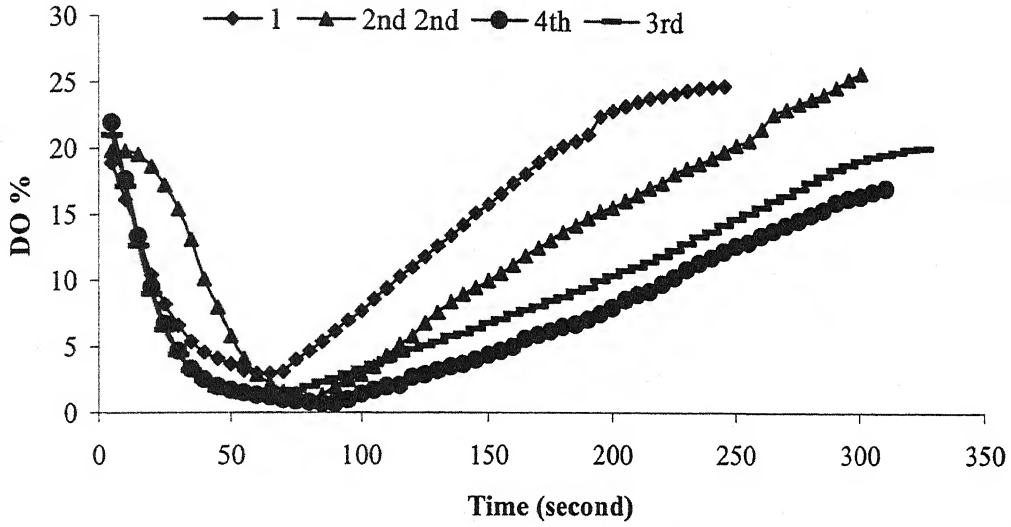


Fig 39. Effect of cell mass concentration on dissolve oxygen concentration profile (DO) on stirred tank reactor

During the 'gassing in' phase, it was observed that the curve became more parabolic in nature. This can also be explained on the basis of increase in the cell mass. As the cell mass increases, it utilizes the more and more of the oxygen being supplied to it. As a result more time is required for the DO level to come back to its initial value and hence the shape of the curve changes (Fig 36)

The KLa values were determined from Fig. and by utilizing equation 4. It can be observed that initially the KLa decreased with the growing the cell mass and then it become stable. (Fig 37)

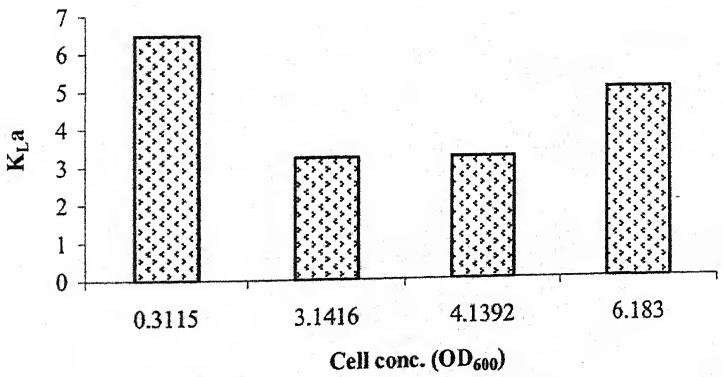


Fig 40. Effect of cell concentration on the volumetric oxygen transfer coefficient (KLa)

4.5. Immobilization studies

4.5.1. Conversion rate of free cells

In order to determine the conversion rate of free cells the reaction was carried out using cell suspension of 40mg/ml in phosphate buffer of 0.1M. The reaction was initiated by adding 30mM of mandelonitrile and samples were collected at regular intervals, conversion was checked by using RP-HPLC and maximum conversion was observed approximately 93%.

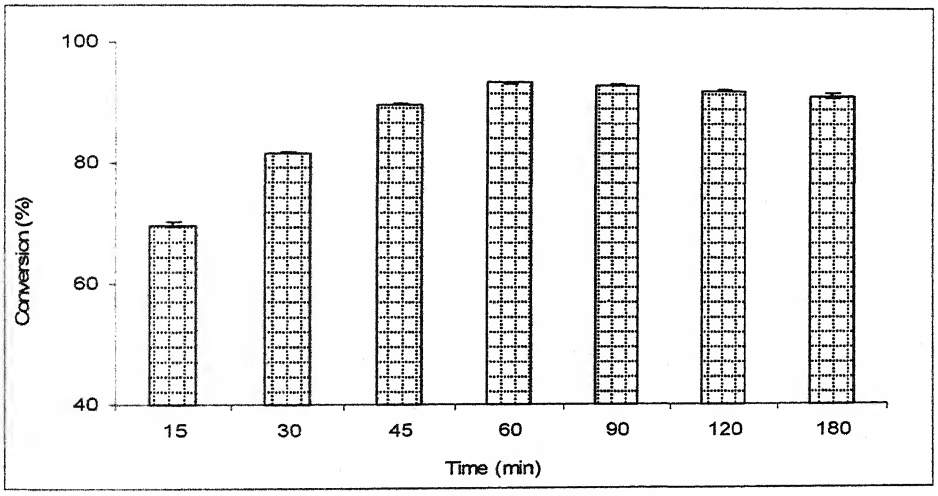


Fig.41. Conversion rate of free cell

4.5.2. Optimization of buffer strength

In order to optimize buffer concentration for immobilization studies various concentrations of tris buffer of (pH 7) were used i.e. (50, 100, 150, 200 mM). The reaction was carried out using these buffers for immobilized cells and checked for maximum conversion using RP-HPLC. It was found that immobilized cells gave maximum conversion with tris buffer of 100mM. This buffer strength was considered optimum and selected for further studies.

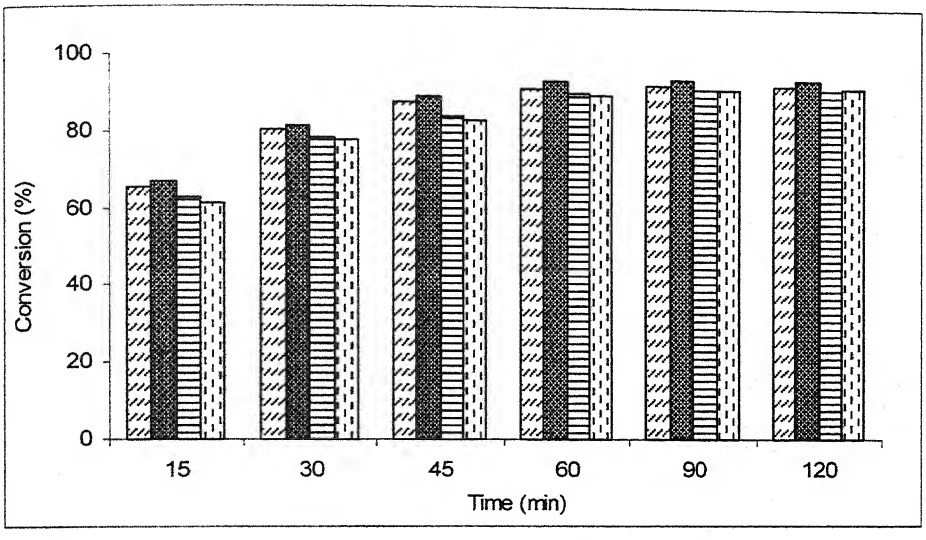


Fig.42. Optimization of buffer strength

4.5.3. Optimization of matrix concentration

In order to optimize the matrix concentration, different concentration of matrix were used ranging from 1.0, 1.5, 2.0 and 2.5, the cells were immobilized using these concentrations and reaction was carried out. With increase in matrix concentration there comes diffusional limitation and with decrease in matrix concentration there is leaching problem. So to overcome this concentration of matrix should be such that it should give maximum conversion with considerable leaching. It was found that beads of sodium alginate formed with concentration of 2% (w/v) were giving good results in terms of conversion and leaching of biocatalyst. This concentration was considered to be optimum and selected for further studies.

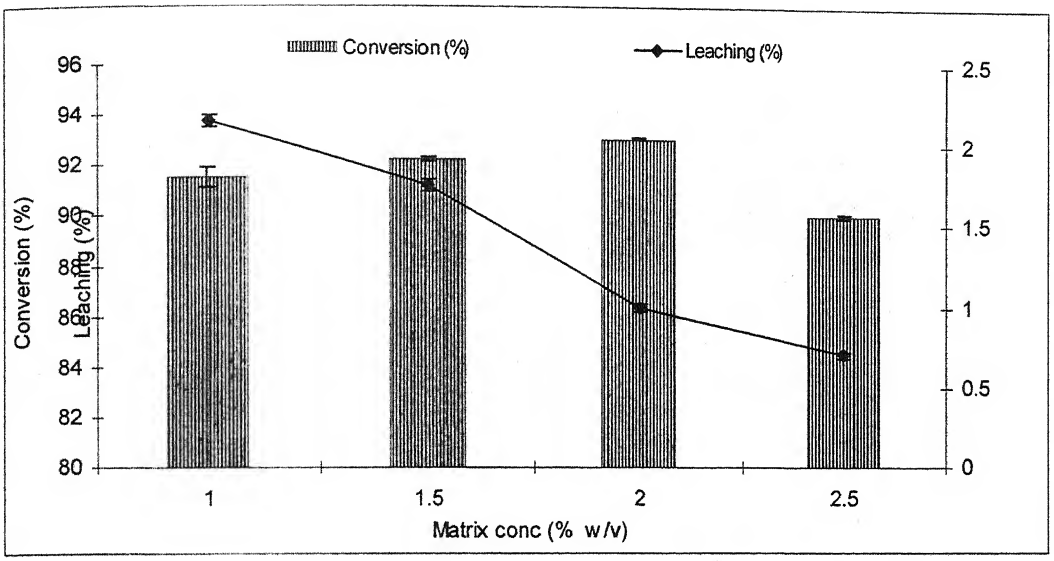


Fig.43. Optimization of matrix concentration

4.5.4. Optimization of cell concentration

For the optimization of cell loading, different amount of cell concentration were used for the immobilization i.e. 5, 10, 20, 40, and 60 mg/ml. With increase in concentration of biocatalyst the rate of reaction increased. Cell concentration 5 mg/ml was found to give conversion of around 86 % and 60 mg/ml was found to give increased conversion with short period of time. The best results were seen with cell concentration of 20 mg/ml. It was found to give optimum conversion comparable with 40 and 60 mg/ml and the cell concentration 20 mg/ml was considered to be optimum and selected for further studies.

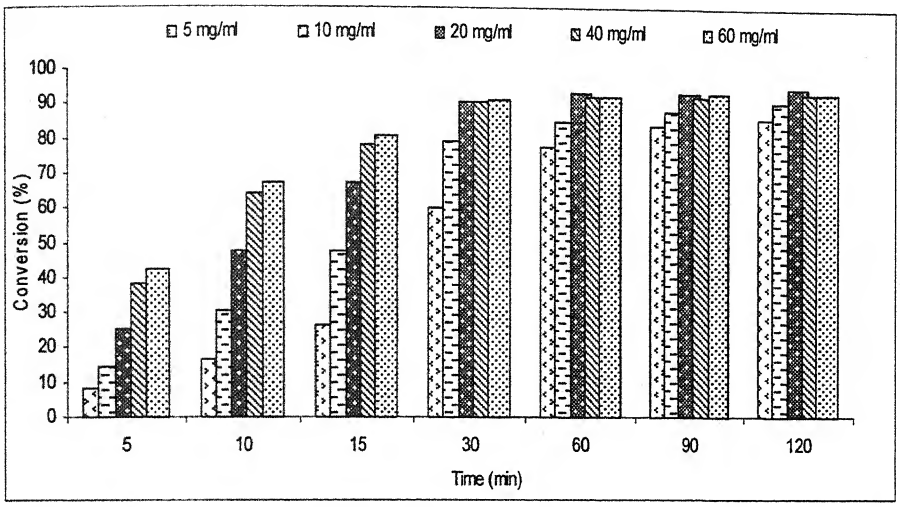


Fig.44. Optimization of cell concentration

4.5.5. Optimization of bead diameter

For the optimization of bead diameter, various size of beads were prepared using sterile syringe i.e. 3.456, 4.30, 4.756, 5.256 and 5.746 mm. The size of the bead should be such that it should provide effective surface area so that the gradient of substrate should be maintained and optimum conversion should be achieved. Optimum conversion rate was found with the bead diameter of 4.3 mm and it is selected for further studies.

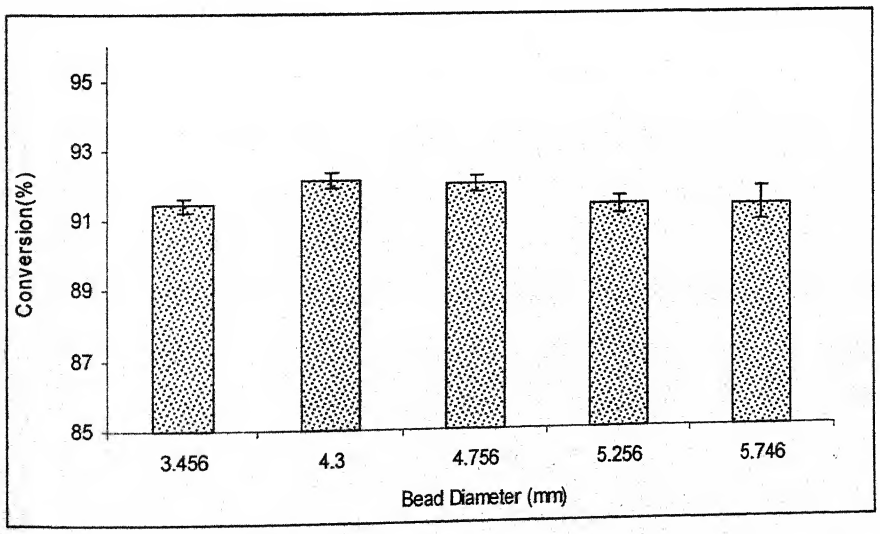


Fig.45. Optimization of bead diameter

4.6. Optimization of physicochemical parameters

4.6.1. Optimization of reaction time

In order to optimize the reaction time, samples were collected at regular interval for 3 hours and checked for conversion using RP-HPLC. It was found that at 60 min the conversion rate was found to be the maximum. Therefore 60 min was selected as optimum reaction time for the further studies.

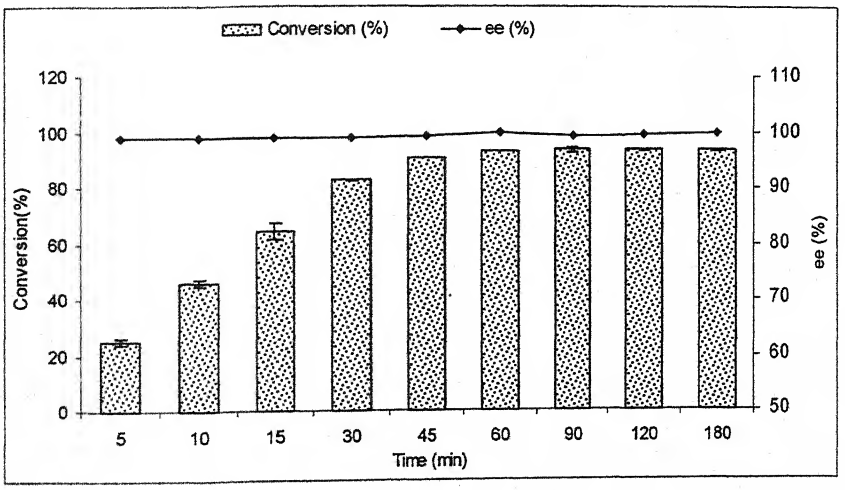


Fig.46. Optimization of reaction time

4.6.2. Optimization of reaction pH

As nitrile hydrolysis follows DKR, it was assumed that pH of the reaction mixture would have profound effect on overall nitrile hydrolysis. The spontaneous decomposition and *in situ* racemization of unreacted mandelonitrile via benzaldehyde and hydrogen cyanide formation occurs at slightly alkaline pH to give 100% therotical yield of *R*-(-)- mandelic acid. Therefore to determine effect of pH on nitrile hydrolysis and to optimize pH, reaction with immobilized cells reaction was carried out at different pH ranging from 6.5 to 8.5. Samples were collected at regular interval till 3 hour and checked for conversion using RP-HPLC. It was found that both at lower and higher pHthe conversion diminished

sharply. Higher conversion was obtained at pH 8 and hence it is selected for further studies.

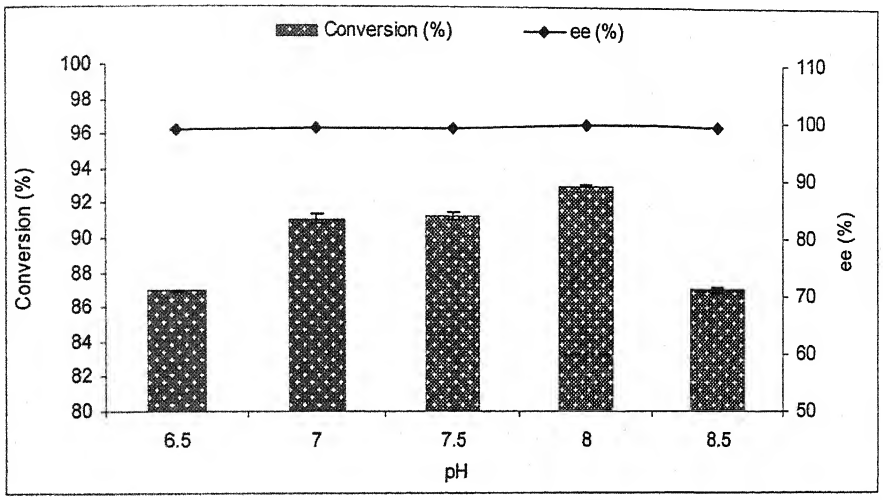


Fig.47. Optimization of reaction pH

4.6.3. Optimization of reaction temperature

The temperature has effect on enzyme activity and enzyme stability therefore it becomes an important parameter in biocatalyric reactions. In order to determine the optimum temperature for the biotransformation, reactions were carried out at different temperatures ranging from 25, 30, 35, 37, 40, and 45 °C and checked for conversion using RP- HPLC. At lower and higher temperature the conversion diminished sharply at temperature 40 °C the conversion was found optimum and it is selected for further studies.

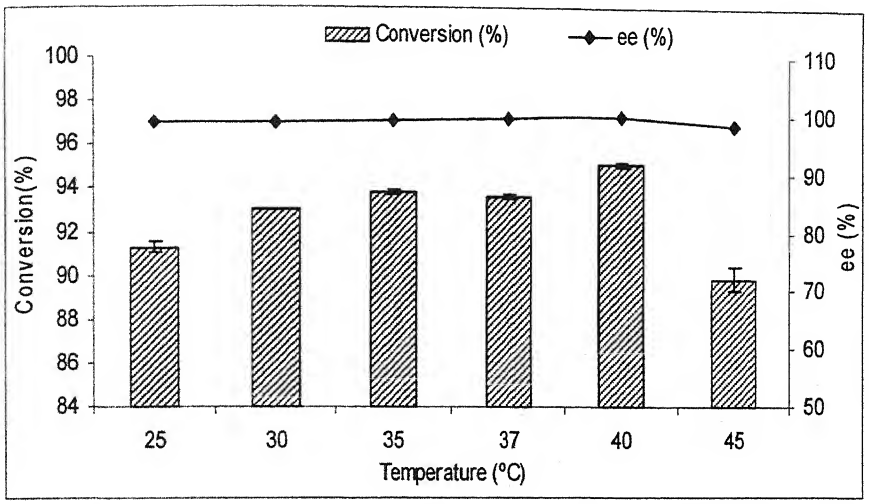


Fig.48. Optimization of reaction temperature

4.6.4. Optimization of substrate concentration

For the optimization of substrate concentration, various amount of mandelonitrile were used i.e. 10, 20, 30, 40, 50, 60, 80 and 100 mM. Samples were collected at regular intervals till 3 hour and checked for conversion using RP- HPLC and it was found that substrate concentration of 50mM was giving the maximum conversion. Above 50mM with the further increase of substrate concentration, the conversion rate decreased. The substrate is sparingly soluble in aqueous medium; therefore, the increased substrate concentration posed diffusion at hindrances decreasing the conversion. Hence substrate concentration of 50mM was found to be optimum and it is selected for further studies.

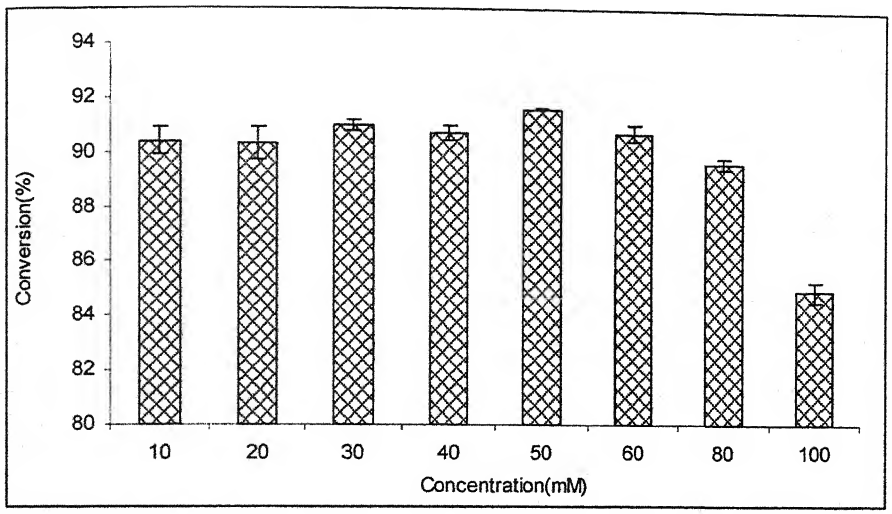


Fig.49. Optimization of substrate concentration

4.7 Optimization of biocatalytic reaction parameters for the enantioselective conversion of mandelonitrile to (*R*)-(-)-mandelic acid

Inspite of the great utility that nitrilases have to offer, their successful utilization on an industrial scale has been overdue. This can be attributed to different properties of the nitrile hydrolytic reaction such as, insolubility of the nitriles in aqueous buffer system, unstable nature of nitrile substrates etc.; as well as properties of the biocatalyst; for example, substrate tolerance, pattern of substrate and product inhibition, biocatalyst stability and recycling etc. Such hurdles can be overcome by adopting different techniques like protein engineering, rational designing, tailor made enzyme preparation and directed evolution. These methodologies provide biocatalysts with much improved properties, however, they are very time consuming, laborious and expensive in nature. Moreover, the instrumentation required to employ these methods are not always easily available. Among many methods to improve the biocatalyst characteristics and synthetic utility, optimization of reaction condition remains a facile and feasible way to achieve the goal. Not only because of the time required but also because of economic feasibility and easier mode of operation, reaction

engineering offers a more attractive alternative compared to other routes. Different biocatalytic reaction parameters were optimized for the efficient bioconversion of mandelonitrile to (*R*)-(-)-mandelic acid with high yield.

4.7.1. Effect of pH

Considering the spontaneous decomposition and *in situ* racemization of unreacted mandelonitrile via benzaldehyde and hydrogen cyanide formation at slightly alkaline pH, it was assumed that pH of the reaction mixture would have profound effect on overall nitrile hydrolysis. Effect of different pH on the conversion of mandelonitrile to mandelic acid was determined by carrying out the reaction in phosphate buffer of different pH's (6 to 8.6). Maximum specific activity was achieved at pH 8. Both at higher and lower pH's the specific activity diminished very sharply. Higher specific activity at this slightly alkaline pH implicated the feasibility of setting up an efficient dynamic kinetic resolution process for the hydrolysis of mandelonitrile to (*R*)-(-)-mandelic acid.

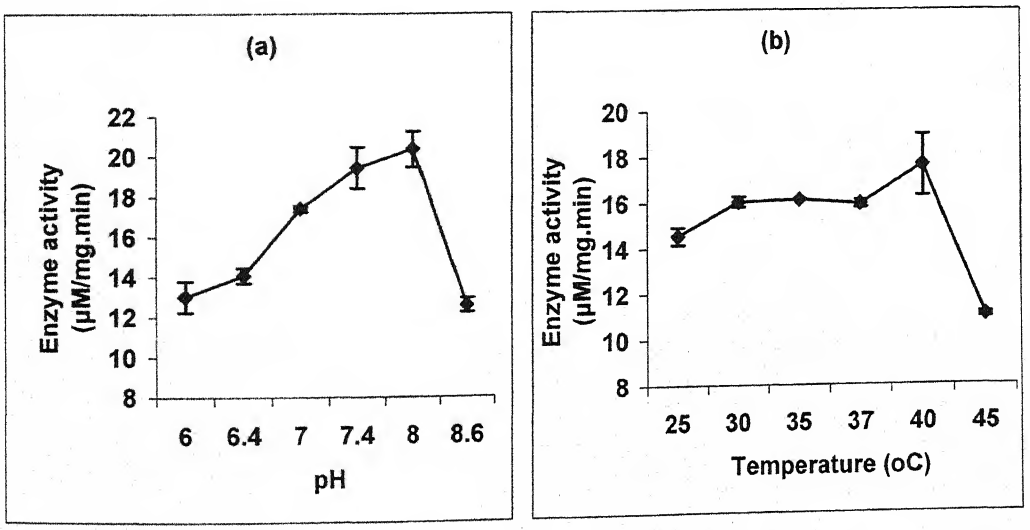


Fig. 50 Optimization of (a) pH and (b) Temperature for nitrilase catalyzed reactions by *E. coli*.

4.7.2 Effect of temperature

Temperature produces opposing effect on enzyme activity and stability and therefore is a key variable in any biocatalytic process. Resting cells of *E. coli* were allowed to react with mandelonitrile at different temperatures ranging from 25 to 45°C and the amount of ammonia formed was estimated. Maximum specific activity was obtained at 40°C. At lower temperature the conversion was very low and at higher temperature probably denaturation of protein rendered the nitrilase inactive.

4.7.3. Effect of cell mass loading

Amount of biomass loading in the reaction mixture also affects the degree of bioconversion as well as the reaction rate. It was observed that with the increase in cell loading enzyme activity increased, ultimately attaining the maximum at 20 mg/ml cell concentration. With further increase in cell concentration there was no change in the amount of mandelic acid formed probably due to mass transfer problems imposed by the concentrated cell slurry which was viscous enough to hinder the availability of the substrate.

4.7.4 Effect of substrate concentration

When resting cells (20 mg/ml) of *E. coli* were exposed to different concentration of mandelonitrile, it was observed that the specific activity increased with increase in substrate concentration and maximum specific activity was achieved at 10 mM of substrate concentration.

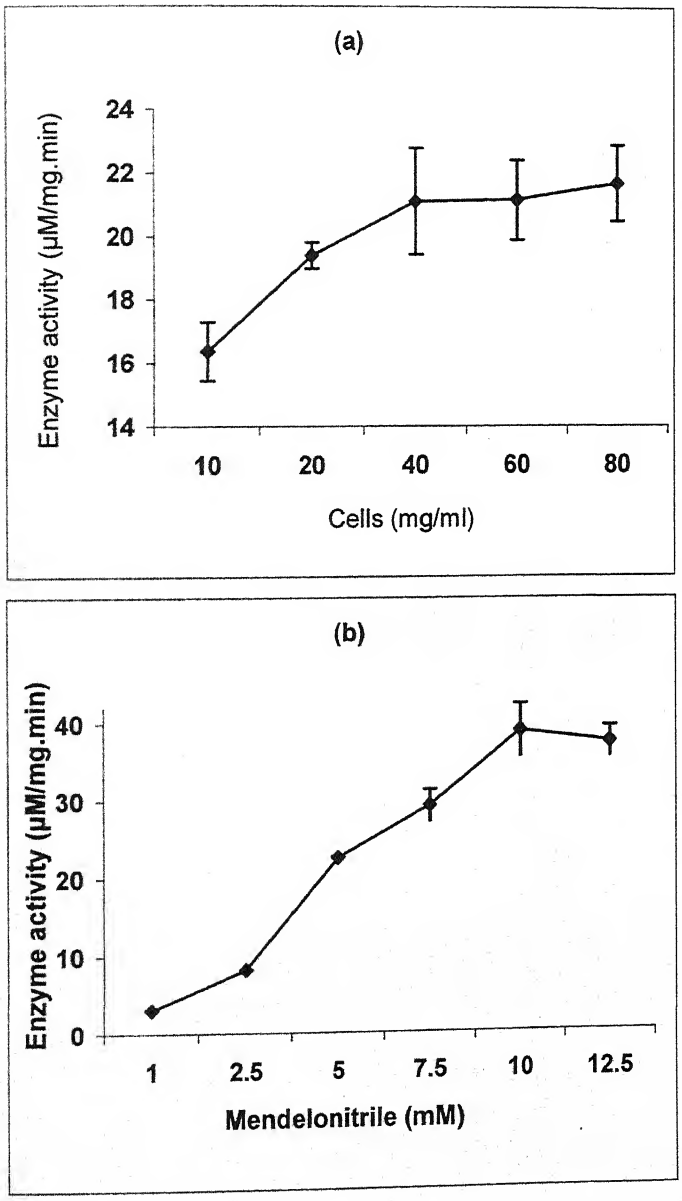


Fig. 51 Optimization of (a) cell loading and (b) substrate concentration for nitrilase catalyzed reactions by *E. coli*.

Conclusion

5. CONCLUSION

The successful utilization of nitrilases for industrial scale production requires an optimized production technology. In this study, stereoselective hydrolysis of mandelonitrile by the recombinant *Escherichia coli* BL21 (DE3) which harbours the nitrilase gene from *Pseudomonas putida* MTCC 5110 was investigated. Nitrilase hydrolyse mandelonitrile to (*R*)-(-)- mandelic acid an important pharmaceutical intermediate as well as a specialty chemical. (*R*)- (-)-mandelic acid is the key intermediate for the production of semi-synthetic cephalosporins, penicillins, antitumor agents, antiobesity agents etc., and is also used as a chiral resolving agent. The condensation polymer of mandelic acid is also being investigated for prevention of entry of human immunodeficiency virus (HIV) and herpes simplex virus through sexual transmission. The molecule has also been shown to possess antifungal, broad spectrum β-lactamase inhibitor and anti-oxidative properties. Optimization of medium components and cultivation conditions remains a facile and feasible way to further enhance the enzyme activity in case of recombinant organism. The favorable characteristics of recombinant *E. coli* implicated large-scale production of nitrilase by this microorganism in a stirred tank reactor. Induces constitutes the major cost for the production of desired product for the recombinant organism. In the first stage we have replaced the costlier inducer i.e. IPTG by a much cheaper sugar (Lactose). After the optimization of the production medium in the shake flask, fermenter studies were commenced where the growth and enzyme activity of the organism without pH control and with pH control were observed. It was seen that the organism gave the best growth and enzyme production when the pH of the medium was controlled to 7.5. Regarding agitation and aeration, the two properties showed direct relation when growth is considered, while an inverse relation is observed when enzyme production is monitored i.e. as we have increased aeration or agitation there is a sharp fall in enzyme activity. Higher agitation and aeration promoted excellent growth, but were found to adversely affect the enzyme activity due to cell lysis by high shear and nitrilase oxidation respectively. Same phenomenon is observed with the feeding experiments, and

hence inducer time was adjusted such that nitrilase production is not hindered. A induction of 0.8% lactose after 21 h of growth was found to be best when the agitation and aeration were adjusted to 200 rpm and 1 vvm respectively.

The stereo selective hydrolysis of nitriles represents pivotal transformation in organic compounds, as products are often useful target bioactive compounds or important intermediates in the synthesis of bioactive compounds. For this reaction, the use of enzymatic protocol offers an alternative to chemical methods and has aroused interest among chemists. At present various enzymes are commercially available; however their use is restricted because of expensive substrates, involvement of co-factors, cost associated with the production of enzymes and above all because of operational stability of the biocatalyst in question.

In this regard, the use of whole cell system is emerging as an economical and eco-friendly alternative. Although operationally simple, the microbial methods often suffer either from limited substrate specificity and/or moderate to good enantioselectivity except in few cases. Thus, there is a need to develop more efficient optimization methods for nitrile hydrolysis. Herein, enzymes, especially nitrilase, offer great potential for enantioselective synthesis. This enzyme possesses broad substrate specificity. In order to overcome the limitation of whole cell, immobilization of whole cell was done to increase stability and reuse of biocatalyst. Immobilization was done by matrix entrapment method with use sodium alginate as matrix. Buffers like phosphate buffer and Tris buffer were used for the immobilization process and Tris buffer (100 mM) was found to give good results and selected for the further studies. Matrix concentration of 2% w/v and cell concentration of 20 mg/ml were found to be optimum. All experiments were done using 30 mM of substrate concentration. The choice of the optimal pH, reaction time and temperature are very important factor for enzymatic synthesis of product. In this work, the influence of reaction time on conversion and the enantioselectivity in the hydrolysis of mandelonitrile was studied and reaction time of 60 min was found to be optimum. The hydrolysis of mandelonitrile by nitrilase from immobilized *Escherichia coli* BL21 (DE3) cells

was examined under different conditions of temperature and pH of reaction mixture. The reaction was giving maximum conversion at a pH 8 and temperature of 40° C. Maximum conversion was found at 50 mM of substrate with 20 mg/ml cell concentration. Making use of these optimization studies, a successful large scale set up was designed for hydrolysis of mandelonitrile with use of immobilized packed bed reactor system and flow rate of 20 ml/h and substrate concentration of 200mM was found to be the optimum.

It may be concluded that the *Escherichia coli* BL21 (DE3) is attractive biocatalyst, capable of carrying out the hydrolysis of mandelonitrile with remarkable stereoselectivity and pivotal biocatalyst for production (*R*)-(-)- mandelic acid an important pharmaceutical intermediate.

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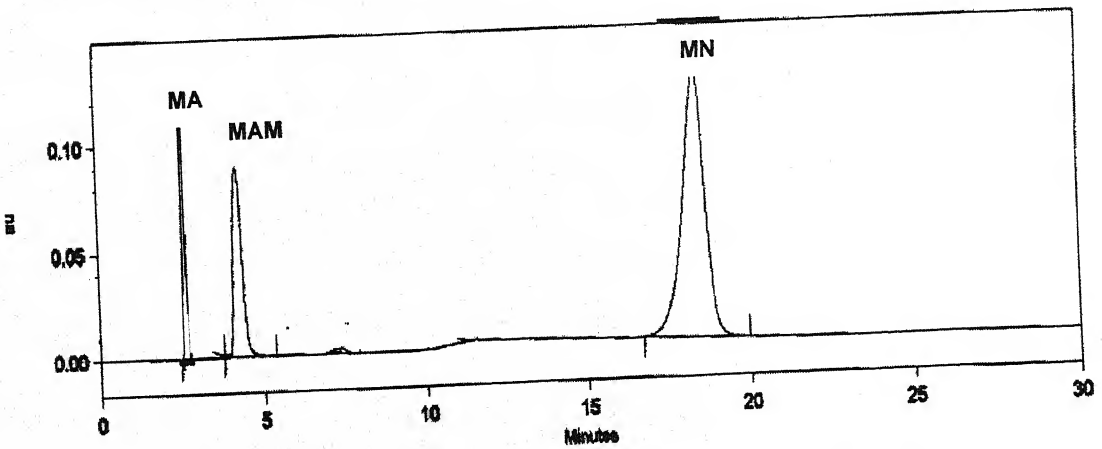
Appendix

8.1. Appendix 1: HPLC chromatograms

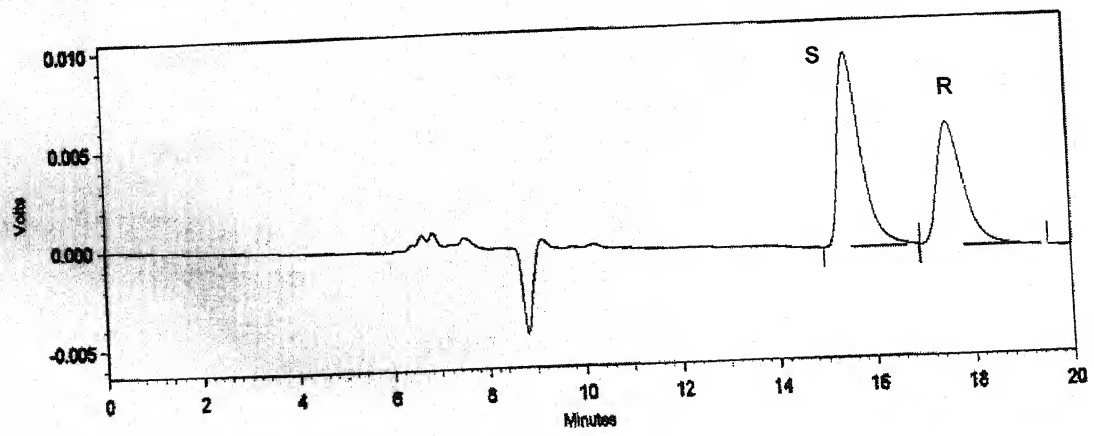
(a) *RP-HPLC chromatogram of mixture of mandelic acid, mandelamide and mandelonitrile*

RP-HPLC chromatogram of mixture of standard mandelic acid (MA, RT-2.6 min), mandelamide (MAM, RT-4.2 min) and mandelonitrile (MN, RT-18.3 min). Mobile phase, phosphate buffer (0.01 M, pH 4.8): Methanol 65:35 (v/v); flow rate, 0.8 ml/min; injection volume, 20 µl; detection wavelength, 254 nm; column used, LiChroCART® RP-18 column (250 x 4 mm, 5 µm) (MERCK, Darmstadt, Germany).



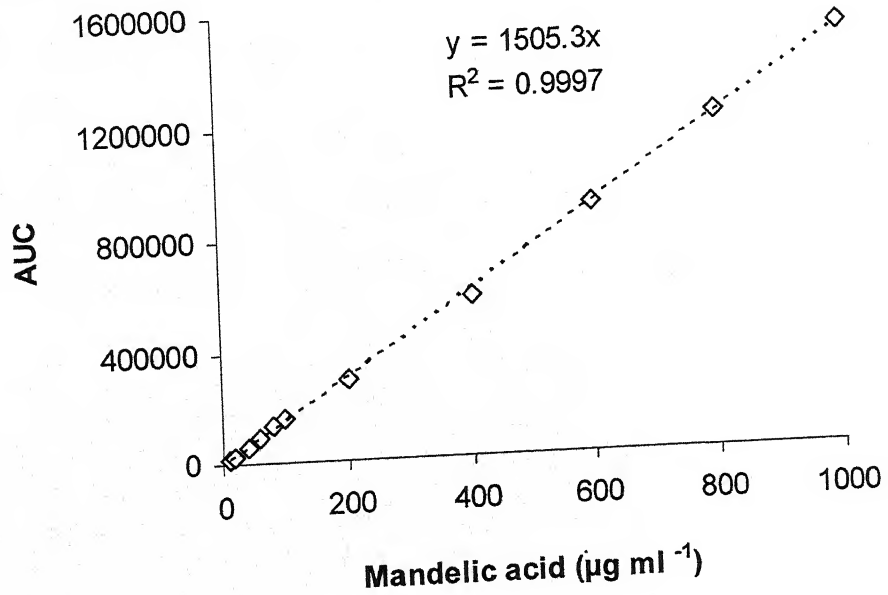
(b) *Chiral-HPLC chromatogram of mixture of R and S mandelic acid*

Chiral-HPLC chromatogram of a mixture of R and S isomers of mandelic acid resolved on a CHIRALCEL-OD-H column (250 x 0.46 mm, 5 µm) (DAICEL Chemical Industries, New Jersey, USA) at a flow rate of 0.5 ml/min using hexane, isopropyl alcohol and tri-fluoro acetic acid (90: 10: 0.2 v/v) as mobile phase. The retention times for S and R enantiomer were 15.5 and 17.5 min respectively.

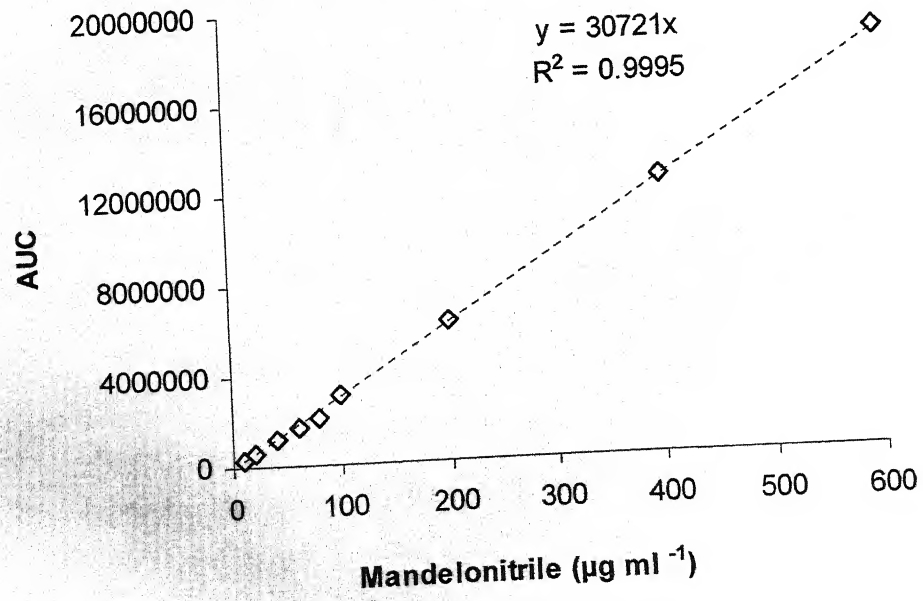


8.2. Appendix 2: Standard graphs

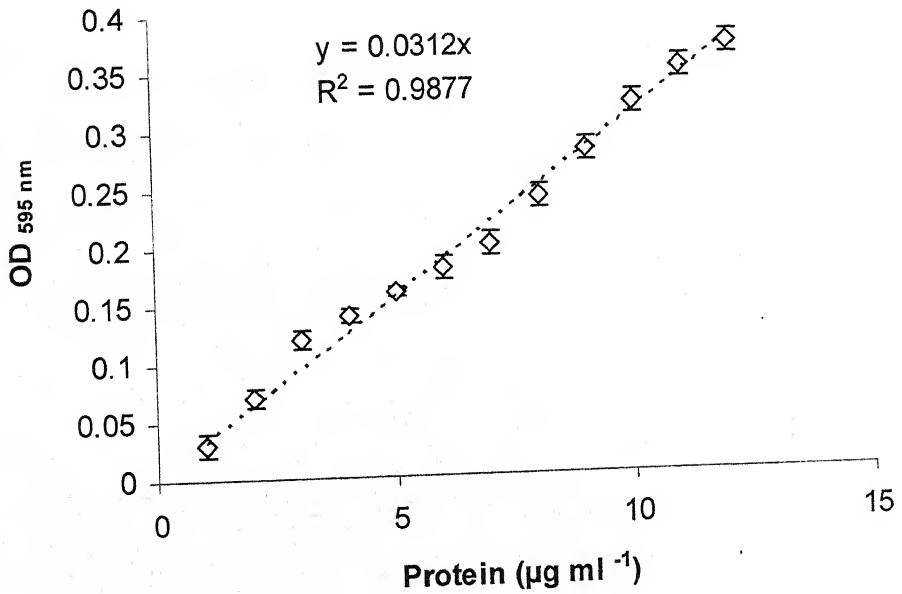
(a) *Standard curve of the mandelic acid by RP-HPLC*



(b) *Standard curve of mandelonitrile by RP-HPLC*



(c) ***Standard curve for estimation of protein by Bradford method***



(d) ***Standard curve for cells mass estimation***